약학박사학위논문

GITR 공자극에 의한 IL-21-producing Follicular Helper T 세포 의존적 항암 면역반응 기전에 관한 연구

Studies on IL-21–producing follicular helper T cell dependent antitumor immune responses triggered by GITR costimulation

2020년 2월

서울대학교 대학원

약학과 의약생명과학 전공

고충현

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지도교수 강 창 율

이 논문을 약학박사학위논문으로 제출함

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2020년 2월

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Abstract

Studies on IL-21–producing follicular helper T cell dependent antitumor immune responses triggered by GITR costimulation

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Although treatment with glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) agonistic antibody (DTA-1) has shown potent antitumor activity in various tumor models, the underlying mechanism is not fully understood yet. Here, I demonstrate that interleukin (IL)-21–producing follicular helper T (Tfh) cells play a crucial role in DTA-

1-induced tumor inhibition. Administration of DTA-1 increased IL-21 expression in an antigen specific manner and this led to enhanced antitumor cytotoxic T lymphocyte (CTL) activity which was mainly produced by Tfh cells. Mice treated with a neutralizing antibody to IL-21 receptor exhibited decreased antitumor activity by DTA-1. Moreover, tumor growth inhibition by DTA-1 was abrogated in *Bcl6*$^{fl/fl}$*Cd4*^{cre} mice, which are genetically deficient in Tfh cells. Mechanistically, IL-4 is required for the optimal induction of IL-21–expressing Th cells by GITR co-stimulation and Bcl6 and c-Maf mediate this pathway. Thus, my findings identify GITR co-stimulation as an inducer of IL-21–expressing Tfh cells and provide a mechanism for its antitumor activity.

Keyword : GITR, IL-21, Follicular helper T cell, antitumor immunity, Cytotoxic lymphocyte, Bcl6

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Abbreviations

αIL-21Rc Ab	anti-Interleukin 21 receptor antibody
Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
Bcl	B cell lymphoma
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocyte
CXCR5	C-X-C Chemokine receptor 5
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
GrzB	Granzyme B
i.p.	Intraperitoneally
i.v.	Intravenously
IFN-ɣ	Interferon gamma
IL	Interleukin
LN	Lymph node
mAb	Monoclonal antibody
ndLN	non draining lymph node

OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed death-1
s.c.	Subcutaneously
TCR	T cell receptor
TdLN	Tumor draining lymph node
Tfh	Follicular helper T
Th	Helper T
TIL	Tumor-infiltrating lymphocyte
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T

Chapter 1. Introduction

1.1. Study Background

Traditional tumor treatment

There were traditional medical treatment against cancer; radiation therapy, surgery, and chemotherapy. Radiation therapy uses high doses of radiation to kill cancer cells and reduce tumors. Radiation therapy causes damages in DNA and slows growth of tumor cells. Cancer cells whose DNA is damaged beyond repair stop dividing or die. When the damaged cells die, they are broken down and removed by body. Surgery is a procedure in which a surgeon removes cancer from patient's body. Surgery works best for solid tumors that are contained in one area. Chemotherapy uses drugs to kill cancer cells. Chemotherapy works by stopping or slowing the growth of cancer cells, which grow and divide quickly. Chemotherapy is used to cure cancer, lessen the chance it will return, or ease cancer symptoms. Although these treatments have medical benefits, each of them has clinical limitations. Radiation not only kills or slows the growth of cancer cells, it can also affect nearby healthy cells. Surgery is restricted to solid tumors and can cause

pains in operated sites and infection. Chemotherapy not only kills fast-growing cancer cells, but also kills or slows the growth of healthy cells that grow and divide quickly. Damage to healthy cells may cause side effects, such as mouth sores, nausea and hair loss. Due to these limitations, researchers found other treatments for cancer patients and immunotherapy is developed.

Immunotherapy

Immunotherapy is a strategy to regulate cancer cells using immune system of cancer patients. The two main strategy of immunotherapy are cellular immunotherapy and antibody immunotherapy. Cellular immunotherapy is divided into Chimeric Antigen Receptor (CAR) T cell therapy, Adoptive T cell therapy (ACT). Antibody immunotherapy is divided into checkpoint blockade therapy and agonistic costimulatory antibody therapy. The importance of immunotherapy has been acknowledged by the *Nobel Prize for physiology or medicine* 2018 awarded for the discovery of *cytotoxic T-lymphocyte-associated protein* (CTLA-4) to James P. Allison and *programmed cell death protein 1* / *programmed cell death protein ligand 1* (PD-1 / PD-L1) to Tasku Honjo (1). Now

immunotherapy has become an established pillar of cancer treatment improving the prognosis of many patients with a broad variety of hematological and solid malignancies **(Figure 1)**. Numerous clinical and preclinical studies about immunotherapy have been reported and significant advances have been achieved.

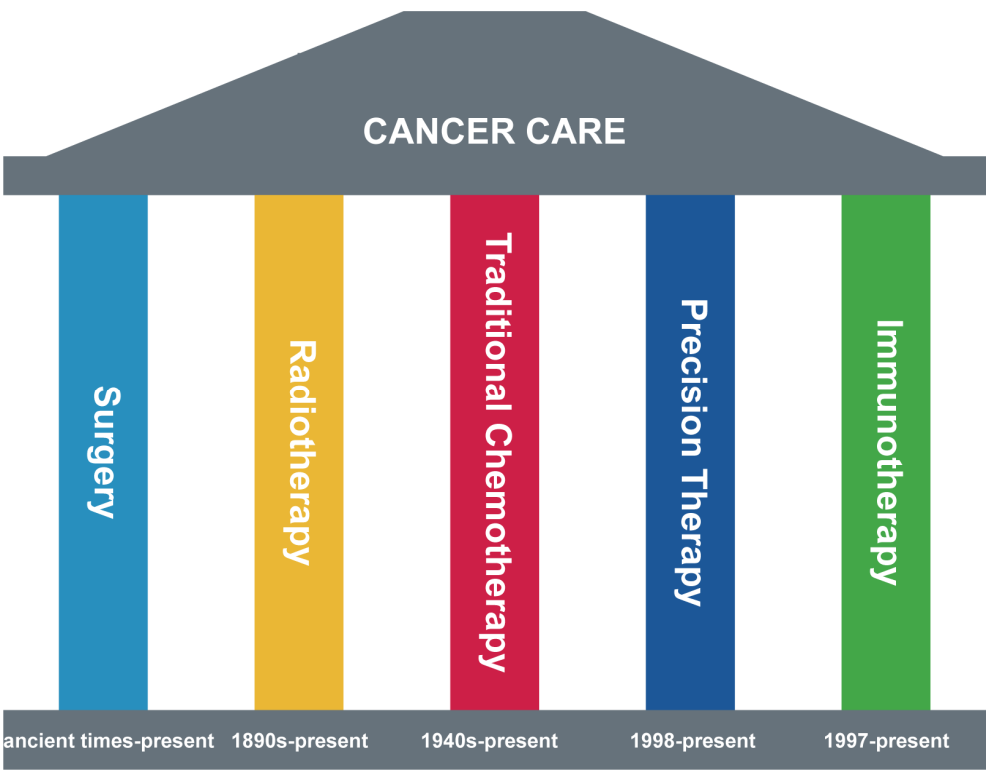

Figure 2. 5 pillars of Cancer Treatment

FDA approved immunotherapeutic medical products are usually utilized

alongside treatment already in use, including surgery, radiotherapy and cytotoxic chemotherapy (2).

Checkpoint inhibitors

Immune checkpoints are regulators of the immune system. These signal pathways are crucial for self-tolerance, which prevents the immune system from attacking healthy cells indiscriminately. In tumor microenvironment, immune checkpoint signals help tumor cells escape from immune surveillance by blocking activation of tumor antigen-specific T cells with CTLA-4 signaling pathways and inhibiting tumor cell killing of tumor antigen-specific T cells with PD-1 signaling pathways **(Figure 2 and 3)**. Checkpoint inhibitors were developed to block the immune escape signals during antitumor immune responses. Checkpoint inhibitors significantly controlled tumor growth in preclinical and clinical models (1,3-6). Due to its effectiveness, intensive studies have been conducted regarding combinational approaches, perioperative uses, new tumor entities, response prediction, toxicity management and use in special patient populations. However, the antitumor effect of checkpoint inhibitor was not able to cover all the cancer patients and researchers found another strategies to care cancer patients.

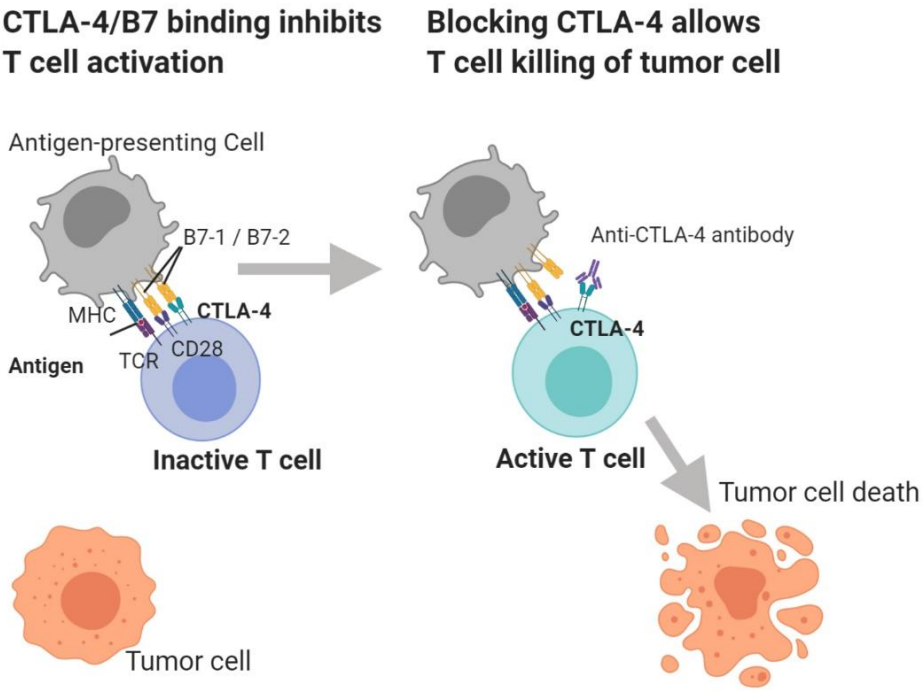

Figure 2. Antitumor mechanisms of CTLA-4 blocking antibody

Checkpoint proteins, such as B7-1 / BB7-2 on antigen presenting cells (APC) and CTLA-4 on T cells, help keep the body's immune responses in check. When the T-cell receptor (TCR) binds to antigen and major histocompatibility complex (MHC) proteins on the APC and CD28 binds to B7-1 / B7-2 on the APC, the T cell can be activated. However, the binding of B7-1 / B7-2 to CTLA-4 keeps the T cells in the inactive state so they are not able to kill tumor cells in the body. Blocking the binding of B7-1/B7-2 to CTLA-4 with an immune checkpoint inhibitor allows the T cells to be

active and to kill tumor cells.

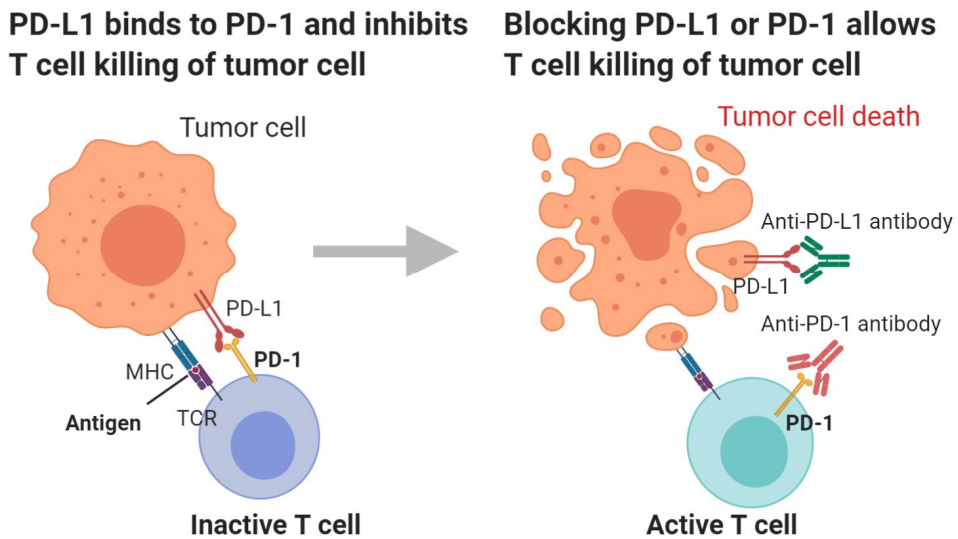

Figure 3. Antitumor mechanisms of PD-1 / PD-L1 signal blocking antibody

Checkpoint proteins, such as PD-L1 on tumor cells and PD1 on T cells, help keep immune responses in check. The binding of PD-L1 to PD-1 keeps T cells from killing tumor cells in the body. Blocking the binding of PD-L1 to PD-1 with an immune checkpoint inhibitor allows the T cells to kill tumor cells

Costimulatory molecule agonistic antibodies

Immune cell function is tightly regulated by costimulatory receptors, which are activated in response to exposure to foreign antigens, and co-inhibitory receptors, which dampen signaling to avoid excessive immune activation, tissue damage and autoimmunity (7). Same goes with antitumor immune responses. Along with immune checkpoints, costimulatory pathways are equally important in driving productive anticancer immunity. There are many different costimulatory molecules in immune cells, including B7 family, the tumor necrosis factor receptor superfamily. CD28 and inducible T cell co-stimulator (ICOS) are costimulatory molecules in B7 family. CD28 is the prototypic costimulatory receptor and a critical mediator of T cell signaling following TCR activation (8). Similarly, inducible T cell costimulatory (ICOS) is another costimulatory receptor important for the function and survival of activated and memory T cells (9,10). The tumor necrosis factor receptor superfamily (TNFRSF) is a large and functionally diverse class of receptors with related structures capable of mediating a range of immune and non-immune cell functions. Of the 29 receptors that are known to belong to this family, 6 receptors have been characterized and

validated to date to have a primary role as immune co-stimulators (TNFRSF5 (also known as CD40), TNFRSF4 (also known as OX40), TNFRSF9 (also known as 4-1BB), TNFRSF7 (also known as CD27), TNFRSF18 (also known as glucocorticoid-induced TNFR-related protein, GITR) and TNFRSF8 (also known as CD30)). These costimulatory molecules are expressed on a number of immune cells including T cells, B cells, NK cells and antigen presenting cells. Because these costimulatory molecules have crucial roles in activation and survival of immune cells, therapeutic agonist agents targeting these costimulatory molecules have been developed (Table 1).

Table 1. Summary of costimulatory agonist antibodies in clinical development

Target	Molecule	Antibody isotype	Company	Stage
CD27	Varlilumab (CDX-1127)	IgG1	Celldex	Phase I/II
CD40	CDX-1140	IgG2	Celldex	Phase I
	SEA-CD40	Non-fucosylated IgG1	Seattle Genetics	Phase I
	RO7009789	IgG2	Roche	Phase I/II
	JNJ-64457107	IgG1	Janssen	Phase I

	(ADC1013)			
	APX-005M	IgG1	Apexigen	Phase I
	Chi Lob 7/4	Mouse/human chimaera IgG1	BioNTech RNA Pharmaceuticals GmbH, University of Southampton	Phase I
GITR	TRX-518	Aglycosyl IgG1	Leap Therapeutics	Phase I
	MK-4166	IgG1	Merck & Co.	Phase I
	MK-1248	IgG4	Merck & Co.	Phase I
	GWN-323	IgG1	Novartis	Phase I
	INCAGN01876	IgG1	Incyte	Phase I/II
	BMS-986156	IgG1	Bristol-Myers Squibb	Phase I/II
	AMG-228	IgG1	Amgen	Phase I
OX40	Tavolimab (MEDI0562)	IgG1	AstraZeneca	Phase I
	PF-04518600	IgG2	Pfizer	Phase II
	BMS-986178	IgG1	Bristol-Myers Squibb	Phase II
	MOXR-0916	IgG1	Roche	Discontinued; phase at termination: phase II clinical
	GSK-3174998	IgG1	GlaxoSmithKline	Phase I
	INCAGN01949	IgG1	Incyte	Phase II
4-1BB	Utomilumab (PF-05082566)	IgG2	Pfizer	Phase II
	Urelumab (BMS-663513)	IgG4	Bristol-Myers Squibb	Phase II
ICOS	GSK-3359609	IgG4	GlaxoSmithKline	Phase I
	JTX-2011	IgG1	Jounce Therapeutics	Phase I
CD28	Theralizumab (TAB-08)	IgG4	TheraMAB	Phase I/II

GITR as a target of antitumor immune responses

As mentioned above, GITR is a costimulatory molecule of the tumor necrosis factor receptor superfamily and is expressed in a broad range of immune cells including T cells, NK cells, and B cells (11). It is well-known that the administration of GITR agonists inhibits tumor growth in various tumor models and CD4 T cells play essential roles in initiating the antitumor immunity induced by the GITR agonistic antibody, DTA-1 (12,13). Treatment with GITR agonists reduced the number and suppressive effects of regulatory T (Treg) cells (14,15). On the other hand, GITR costimulation increased the number of effector CD8 T cells in the tumor microenvironment and upregulated their cellular metabolism (16-18). Recently, the first in-human phase 1 trial of GITR agonistic antibodies (TRX518) was performed and the safety profile and immune effects in the patients were reported (19).

Role of IL-9 in antitumor immune responses of DTA-1

Previous study in our lab demonstrated that IL-9 plays a fundamental role in the antitumor activity of GITR agonistic antibody, DTA-1 (20). In this study, several mechanisms of antitumor immune responses of DTA-1

were revealed. First, GITR costimulation promoted the differentiation of naïve CD4 T cells into antitumorigenic Th9 cells rather than protumorigenic regulatory T (Treg) cells. Therefore, GITR costimulation shifted the balance of CD8 to Treg ratio in tumor tissue and contributed antitumor effect of DTA-1. Moreover, GITR costimulation induced IL-9 activated tumor antigen specific cytotoxic T lymphocytes via stimulation of dendritic cells **(Figure 4)** (21). In addition to the discovery with IL-9, previous researcher also found that DTA-1 treatment induced IL-21 expression in CD4 T cells, and questions arose concerning whether IL-21 mediates the antitumor immunity that is induced by DTA-1.

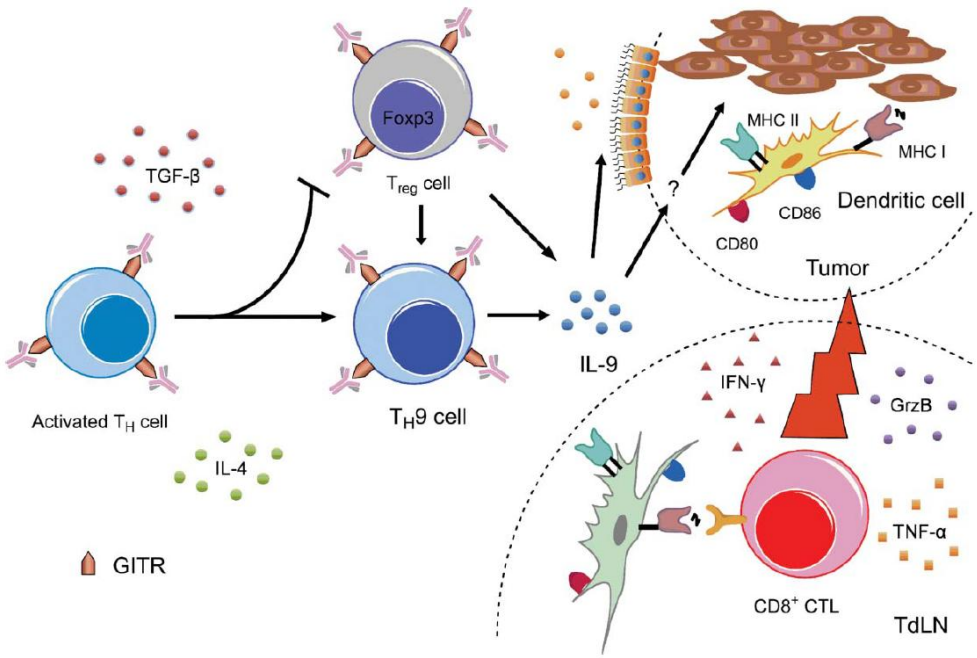

Figure 3. A schematic illustration of Th9 cell-mediated antitumor immunity induced by GITR costimulation.

GITR triggering inhibits iTreg cell generation and promotes Th9 cell differentiation. IL-9 production triggers epithelial cells to chemoattract dendritic cells into the tumor and enhances the cross-presentation and costimulatory capacity of the tumor-infiltrating dendritic cells. These tumor-antigen-crosspresenting dendritic cells then potentiate tumor-specific $CD8^+$ CTL responses, thereby facilitating tumor regression (21).

Role of IL-21 in antitumor immunity

IL-21 is a member of the common-γ-chain cytokine family which includes IL-2, -4, -7, -9, and -15 (22). IL-21 is produced by NKT cells, Th17 cells, and follicular helper T cells(Tfh cells) (22) and potently stimulates B cells, NK cells, and CD8 T cells. IL-21 is a pleiotropic cytokine and well known for its antitumor activities. Treatment with recombinant IL-21 has been shown to induce NK cell-mediated antitumor activity via NKG2D (23). In addition, IL-21 induced the expansion of cytotoxic CD8 T cells without the collateral expansion of Treg cells, a role of which is different from IL-2 (24). In breast cancer patients, $CCR4^{-}CCR6^{-}CXCR3^{-}$ $CD4^{+}$ T cells were expanded in the peripheral blood and promoted the cytotoxicity caused by autologous CD8 T cells via IL-21 (25). Furthermore, a combination of recombinant IL-21 with rituximab showed a synergistic effect on tumor regression in cynomolgus monkeys and improved antibody dependent cellular cytotoxicity (ADCC) in human NK cells *in vitro* (26). With PD-1 blockades, IL-21 exhibited potent antitumor activity against H22 murine hepatocellular carcinoma by increasing CTL-induced cytotoxicity (27). Previous study in our lab has also shown that IL-21 exerts antitumor activity by restoring the function of $TIM\text{-}3^{+}PD\text{-}1^{+}$-exhausted NK cells in MHC

class-I deficient tumors (28).

Follicular Helper T (Tfh) cells

T cell help to B cells is a fundamental aspect of adaptive immunity and the generation of immunological memory. Follicular helper CD4 T (Tfh) cells are the specialized providers of B cell help. Tfh cells were recently defined CD4 T cell subset. Tfh cells depend on expression of the master regulator transcription factor Bcl6. Distinguishing features of Tfh cells are the expression of CXCR5, PD-1, SAP (SH2D1A), IL-21, and ICOS, among other molecules, and the absence of Blimp-1 (*prdm1*). Tfh cells are important for the formation of germinal centers. Once germinal centers are formed, Tfh cells are needed to maintain them and to regulate germinal center B cell differentiation into plasma cells and memory B cells **(Figure 5)** (29). Even though the master transcription factor for Tfh cells has not been defined yet, Bcl6 is considered as a key transcription factor for Tfh cell development (30-32). Tfh cells are essential for the generation of most isotype switched and affinity matured antibodies, and therefore they have an obvious role in protective immunity against pathogens (33). Antibodies are necessary for the control of LCMV infection, and defects in Tfh cells result

in failure to control LCMV (34). Tfh cell frequencies are associated with the amount and quality of antibody responses against SIV (simian immunodeficiency virus, a close relative to HIV) in SIV-infected macaques (35). Impaired Tfh cell help to B cells is observed in HIV-infected individuals, which appears to exacerbate the difficulty in generating neutralizing antibodies against HIV (36). Tfh cells are central players in a number of autoimmune diseases, and it is hoped that a greater understanding of Tfh cells can result in new therapeutic approaches against major autoimmune diseases. Increased frequencies of Tfh-like cells in peripheral blood are observed in subsets of patients with Sjogren's syndrome (37), juvenile dermatomyositis (38) and systemic lupus erythematosus (39). Tfh cells might contribute to autoimmune diseases both by facilitating aberrant generation of autoantibodies and by facilitating the formation or maintenance of ectopic follicles, which serve as nucleation points for other cells that might be directly pathogenic in the autoimmune disease.

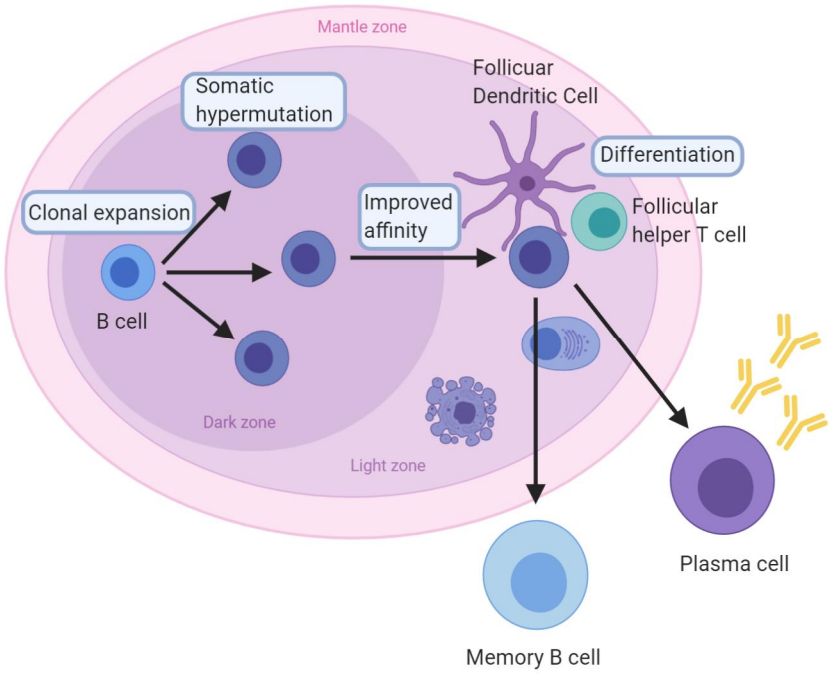

Figure 4. Germinal center structure and follicular helper T cells

Tfh cells help B cells via cytokines and cell to cell contact interactions. In addition, Tfh cells likely provide signals to follicular dendritic cells, the third cell type of germinal center triumvirate. Tfh cells are required for the formation and maintenance of germinal centers and for the generation of B cell memory. The control of these processes hinges on Tfh regulation of multiple B cell fate decision, including cell death.

Tfh cells and its role in antitumor immunity

Althought roles of Tfh cells are expected in allergy, autoimmune disease and infectious diseases, roles of Tfh cells in tumor immunity is still controversial (40-42). Tumor infiltrating Tfh cells were positively correlated with clinical outcomes in breast cancer patients with development of ectopic lymphoid organ-like structure (40). In human colorectal cancer, similar results were observed (43). However, IL-4 produced by Tfh cells is shown to compromise antitumor immunity through accumulation of $CD11b^{+}$ immunosuppressive myeloid cells in the tumor microenvironment, and Tfh cells have protumorigenic activity in diffuse large B cell lymphoma (DLBCL) via IL-10 (41,42).

1.2. Purpose of Research

Several mechanisms of antitumor immune responses of GITR costimulation were demonstrated. The initiative roles of CD4 T cells in GITR agonistic antibodies were elucidated in mice tumor models (12). GITR targeting antibodies directly bind to Treg cells which highly express GITR on cell surface and downregulates the frequency of Treg cells (13). Mature B cell-deficient JHD mice showed impaired antitumor effect of GITR agonistic antibodies indicating the critical role of B cells in these antitumor immune responses (44). Further studies described the detailed mechanisms of GITR costimulation in aspects of differentiation of IL-9 producing–CD4 T cells that results in balance shifting of protumorigenic to antitumorigenic circumstances (20,21). In addition to IL-9, GITR costimulation enhanced production of IL-21 which is well-known for its antitumor effects (20,21).

In this study, I tried to demonstrate the role of IL-21 in antitumor immune responses of GITR costimulation using DTA-1, GITR agonistic antibody. First, I examined the tumor growth of DTA-1 treated mice with inhibition of IL-21 signal. Next, I tried to find out which cells produced IL-

21 after DTA-1 treatment. After I found the IL-21 producer, I asked cellular and molecular mechanisms of IL-21 production. Lastly, I tried to demonstrate the requirement of IL-21 producing cells in antitumor immune responses of DTA-1 using chemical inhibitor and conditional knockout mice.

Chapter 2. Materials and Methods

Mice

Six- to eight week-old female C57BL/6 and BALB/c mice were purchased from the Charles River Laboratories. Bcl-$6^{fl/fl}$ mice, OT-I mice and OT-II mice were purchased from the Jackson laboratory. The CD45.1 and IL-$4Ra^{-/-}$ mice were kindly provided by J.-O. Kim(International Vaccine Institute) and Y.-K. Kim, respectively. The CD4-cre mice were kindly provided by Y.-S. Chung. All mice were maintained under specific pathogen-free conditions in the animal facility of the Pharmaceutical Research Institute at Seoul National University. All animal studies strictly adhered to the approved Institutional Animal Care and Use Committee protocols (SNU-181214-1) at Seoul National University.

Tumor cell lines

The CT26 (purchased in 2002), TC-1 (purchased in 2006) and B16F10 (purchased in 2016) cells were purchased from the ATCC and MC38 cell lines were kindly provided from Genentech in 2018. These cell lines were cultured in DMEM (Gibco) supplemented with 10% FBS and 1%

penicillin-streptomycin (DF10). The B16F10-OVA cells (kindly provided in 2006 by Dr. K. Rock, University of Massachusetts Medical School, Boston, MA) were cultured in DF10 supplemented with 200 μg/ml of geneticin (Gibco) and 60 μg/ml of hygromycin (Invitrogen).

Antibodies and reagents

The fluorochrome conjugated antibodies to mouse CD3ε(145-2C11), CD4(RM-4-5), CD8α(53-6.7), CXCR5(L138D7), Streptavidin, anti-human IgG(HP6017), IFN-γ(XMG1.2), TNF-α(MP6-XT22), CD107a(1D4B), CD138(281-2), CD25(PC61), CD44(IM7), CD62L(MEL-14), CD45.1(A20) and CD45.2(104) were purchased from BioLegend. The antibodies to mouse PD-1(J43), GL-7(GL7), CD95(15A7) were purchased from eBioscience. The mIL-21R-hIgG-Fc was purchased from R&D systems. The 79-6 was purchased from Calbiochem. The CFSE were purchased from Invitrogen.

Antibody staining and flow cytometry analysis

Cells were stained with dye-conjugated antibodies in 1% FBS and 0.02% azide containing PBS buffer. For intracellular staining, cells were restimulated with brefeldin A(GolgiPlug; BD Biosciences) and monensin

(GolgiStop; BD Biosciences) for 4 hours. Cytofix-Cytoperm kits (BD Biosciences) were used for fixation and permeabilization according to the manufacturer's instructions. An antibody against CD107a was added during restimulation. For staining transcription factors, cells were fixed and permeabilized with a Foxp3 staining kit (eBioscience).

Transplant tumor models

A total of 2×10^5 CT26 cells were subcutaneously (s.c.) injected into the left flank of the BALB/c mice, and 2×10^5 TC-1, MC38 and B16F10 cells were subcutaneously (s.c.) injected into the left flank of the C57BL/6 mice. Five days later, 600 μg of DTA-1 or the rIgG2b control (LTF-2; BioXcell) was intraperitoneally (i.p.) administered. For blocking IL-21 signaling, 300 μg of anti-mouse IL-21 receptor (4A9; BioXcell) or the rIgG2a control (2A3; BioXcell) was administered every 2 or 3 days starting 8 days after tumor inoculation. For the Bcl6 inhibition, 1 mg of 79-6 (Calbiochem) was i.p. injected daily between 8 and 14 days after the tumor challenge. $Bcl6^{fl/fl}Cd4^{Cre}$ mice were s.c. injected with 2×10^5 TC-1 cells, and 600 μg of DTA-1 was i.p. administered. The tumor sizes were measured with calipers every 2 or 3 days when the tumor cells were palpable.

In vitro CTL using Calcein-AM

Eight days after DTA-1 injection, the spleen and tumor-draining lymph nodes from CT26 tumor bearing mice were harvested and cultured with the CT26 epitope AH-1 for 5 days. The CT26 cells were labelled with 1 μM Calcein-AM (C3100MP; Life Technologies) and cocultured with AH-1 stimulated effector cells for 4 hours. Using a SpectraMAX M5 (Molecular Devices), the fluorescence of the supernatant from the wells with cocultured cells was analyzed.

Immunohistology staining of tumor draining lymph nodes

Lymph nodes were obtained from CT26 tumor bearing mice 7 days after DTA-1 treatment. Obtained lymph nodes were cryoreserved in OCT compound. Cryoreserved lymph nodes were 9 μm sectioned using Cryostat Microm HM 525 (Thermo Scientific). Sectioned lymph nodes were located on slide glasses and fixed with acetone for 30 min at -20°C. After fixation, Samples were placed in PBS for 30 min at room temperature. Remaining

PBS were carefully removed and samples were blocked with blocking buffer (0.3% BSA and 0.1% tween in PBS). Fixed samples were stained with 100X anti-B220 PE, 400X anti-PNA FITC and anti-CD4 APC antibodies at 4°C overnight in the humidifying chamber. On the next day, stained samples were washed with wash buffer (0.1% BSA and 0.1% tween in PBS) for 3 times. After washing, 1 – 2 drops of Gold solution (Sigma) were used for developing. Immunohistology images were obtained with Confocal scope TC58 (LEICA).

Differentiation of IL-21–producing CD4 T cells in vitro

$CD4^+CD25^-CD44^{lo}CD62L^{hi}$ naïve T cells or $CD4^+CD25^-$ T cells were purified by flow cytometry and stimulated for 4 days with plate-bound anti-mouse CD3ε (2 μg/ml, 145-2C11; BioLegend) and anti-mouse CD28 (1 μg/ml, 37.51; BioLegend) or T cell-depleted splenocytes (1 : 5), supplemented with IL-2. To induce IL-21, IL-4 (10 ng/ml; R&D Systems), IL-6 (10 ng/ml; Peprotech), IL-12 (10 ng/ml; Peprotech), and IL-27 (10 ng/ml; Peprotech) were added to culture well of naïve CD4 T cells. For some experiments, 2 μg of anti-mIL-4 (11B11; ATCC), anti-mIL-6

(MP520F3; R&D Systems), anti-mIL-12/IL-23p40 (C17.8; R&D Systems), and anti-mIL-27 p28/IL-30 (AF1834; R&D Systems) were added during culture. The accumulated supernatants were collected from the 4-day culture, and the IL-21 concentration was determined using an IL-21 ELISA kit (Invitrogen) according to the manufacturer's instructions.

Preparation of tumor infiltrating lymphocytes

Tumor tissues from tumor bearing mice were cut into small pieces and dissociated with gentleMACS Dissociator (Miltenyi Biotec). The dissociated tumor tissues were digested in 2% FBS RPMI medium containing 1 mg/ml collagenase D (Roche), 100 μg/ml hyaluronidase (Sigma Aldrich), and 100 μg/ml DNase I (Sigma Aldrich) at 37℃ for 30 min. From the digested tumor tissues, the lymphocytes were isolated by lymphocyte separation medium (MP Biomedicals) and used for experiments.

c-Maf silencing in CD4 T cells

The scrambled vector used as a control and the c-Maf shRNA vector were kindly provided by S.-H. Im.(Division of Integrative Biosciences and Biotechnology, Department of Life Sciences, Pohang

University of Science and Technology (POSTECH). Retroviral supernatants were generated by Platinum-E (Plat-E) retroviral packaging cell lines with FuGene HD transfection reagent (Promega) according to the manufacturer's instructions. FACS sorted naïve CD4 T cells were stimulated with plate-bound αCD3 (2 μg/ml) and αCD28 (1 μg/ml) with 10 ng/ml of IL-2 for 24 hours and the cells were transduced with retroviral supernatants containing 4 μg/ml of polybrene by means of spin infection (800 g for 90 min at 37°C). After infection, the CD4 T cells were cultured in the presence of plate-bound αCD3 (2 μg/ml) and αCD28 (1 μg/ml) with IL-4 (10 ng/ml) and DTA-1 (2 μg/ml). Eighteen or forty eight hours later, the GFP^+ cells were sorted and resuspended with TRIzol reagent (Invitrogen) for qPCR analysis.

Preparation of human PBMC

Human peripheral blood cells were obtained from healthy volunteers and informed consent was received from all donors. Mononuclear cells were isolated by Ficoll-Histopaque (Sigma Aldrich) density gradient centrifugation. The collection of human samples and all human experiments were approved by the ethical committee of Seoul National University (IRB No. 1712/001-003).

Adoptive transfer model

1:1 mixture of CD4 T cells (2×10^6 each) from the $CD45.1^+CD45.2^+$ OT-II mice and $CD45.1^+$ B6/SJL mice was intravenously injected into the B16F10-OVA tumor-bearing C57BL/6 mice 9 days after tumor challenge. The next day, 600 μg of DTA-1 or rIgG2b control antibody was administered, and the tumor-draining lymph nodes were analyzed 7 days after antibody treatment.

Quantitative PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed using AmfiRivert cDNA Synthesis Platinum Master Mix (GenDEPOT). The synthesized cDNA was quantified with a TB Green™ Premix Ex Taq™ (TaKaRa) and the AriaMx 96 Real-Time PCR System (Agilent). The expression levels of the target genes were normalized to the amount of *mHprt* expression.

Immunoblot analysis

The cytoplasmic fractions of the cells were prepared as follows: *in vitro* cultured CD4 T cells were washed once with ice-cold PBS and collected by centrifugation at 5,000 r.p.m. for 10 min. The cells were resuspended in RIPA buffer (R0278, Sigma-Aldrich) with 1 mM DTT and 0.25 mM PMSF, and a proteinase inhibitor cocktail. The mixtures were vortexed at high speed for 30 min. The extracts were collected by centrifugation at 13,000 r.p.m. for 10 min. The supernatants were collected as cytoplasmic extracts. These cell extracts were loaded onto SDS-PAGE gels and then transferred to PVDF membranes using iBlot 2 PVDF Mini Stacks (Invitrogen). The membranes were stained with specific antibodies and chemiluminescence was visualized using an LAS-3000 LuminoImage analyzer (Fuji film).

Statistical analysis

Unpaired two-tailed Student's t tests and two-way ANOVA were used for statistical analyses. The results with a P value of < 0.05 were considered statistically significant. Data are presented as the means ± s.e.m.

Chapter 3. Results.

IL-21 signaling is essential for DTA-1-induced antitumor immunity by potentiating CD8 T cell responses.

To examine the role of IL-21 in DTA-1-induced antitumor immune responses, I used a well-established tumor models; CT26, TC-1 and MC38. BALB/c mice were subcutaneously injected with CT26 cells and were given DTA-1 or rIgG2b as a control 5 days after tumor inoculation. Consistent with earlier studies (12,13,20,44), the DTA-1 treatment remarkably suppressed tumor growth of the CT26 cells; however, administration of the anti-IL-21 receptor (αIL-21R) significantly abrogated this tumor inhibitory effect in CT26, TC-1 and MC38 model **(Figure 5)**.

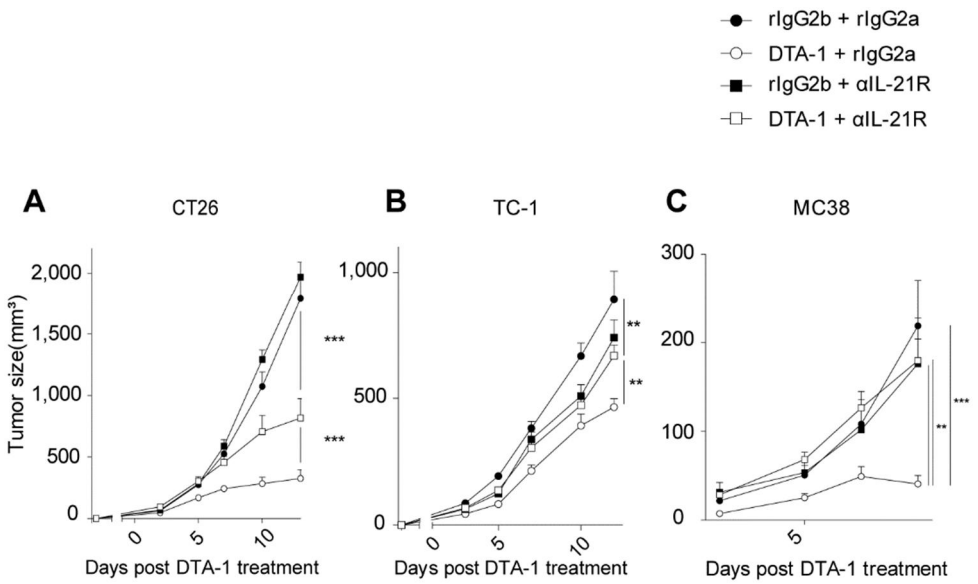

Figure 6. Blocking IL-21 signaling compromises antitumor activity of DTA-1.

(A, B and C) Balb/c or C57BL/6 mice were subcutaneously injected CT26 **(A)**, TC-1 **(B)** or MC38 **(C)** tumor cells. 5 days after tumor inoculation, mouse were intraperitoneally injected with DTA-1 or control rIgG2b antibodies. 2 days later, mice were intraperitoneally injected with αIL-21R and control rIgG2a antibodies every 2 to 3 days (n=8-9 per each group). Tumor size were monitored since 2 days after antibody administration. **(A, B and C)** The data represent at least two experiments. Results were shown as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P< 0.005$ as determined using two way ANOVA.

Next, I investigated the cellular mechanism by which DTA-1 administration triggers antitumor immunity via IL-21. Given that NK cells and CD8 T cells play a key role in eliminating tumors and are also known as targets of IL-21 (45-50), I analyzed the production of effector molecules by CD8 T cells and NK cells in CT26 tumor bearing mice. Eight days after the DTA-1 treatment, production of IFN-ɣ and granzyme B by NK cells were unchanged **(Figure 7)**.

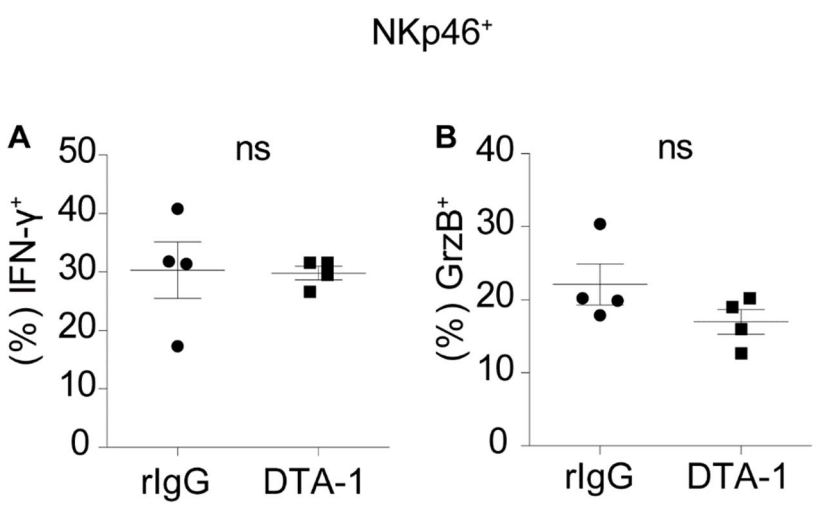

Figure 7. DTA-1 does not affect effector molecule secretion from NK cells.

Balb/c mice were subcutaneously injected CT26 tumor cells. 5 days after tumor inoculation, mice were intraperitoneally injected with DTA-1 or control rIgG2b antibodies. 8 days later, IFN-ɣ **(A)** and Granzyme B **(B)** production in NK cells from TdLN were analyzed using flow cytometry. **(A and B)** The data represent at least two experiments. Results were shown as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P< 0.005$ as determined using a Student's t-test.

On the other hand, the expression of effector molecules and degranulation marker was upregulated by DTA-1 in the CD8 T cells, but was subsequently downregulated by αIL-21R treatment **(Figure 8)**.

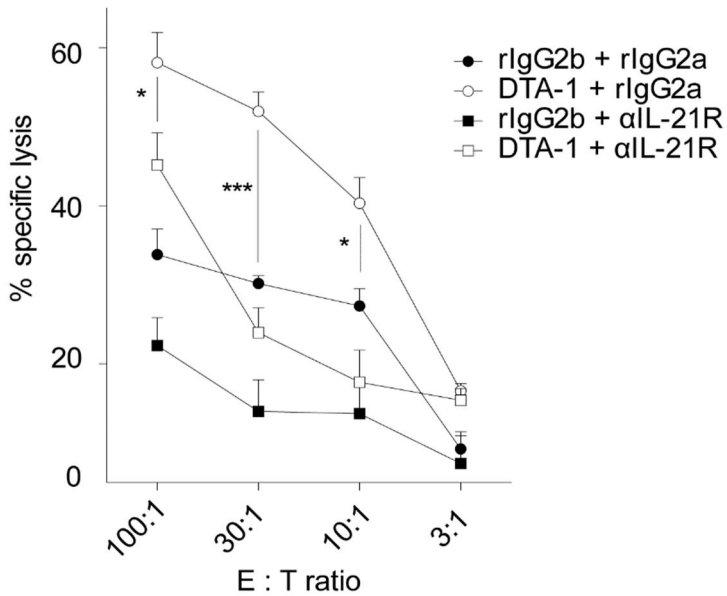

Figure 8. DTA-1 induced IL-21 potentiates CTL responses.

CT26 tumor bearing mice were treated with DTA-1 or αIL-21R antibodies. 8 days after DTA-1 treatment, spleen and tumor draining lymph node (TdLN) cells were harvested and stimulated with CT26 epitope AH-1 for 5 days. Stimulated effector cells (E) were co-cultured with Calcein-AM labelled CT26 cells (T) for 4 hours with indicated ratio. The percentage of specific lysis were calculated with the fluorescence from harvested sup. The data represent at least two experiments. Results were shown as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P< 0.005$ as determined using two way ANOVA.

Consistently, I observed a potent target cell killing activity of CD8 T cells from the DTA-1-treated mice which was significantly decreased by αIL-21R treatment **(Figure 96)**. In comparison with other immune checkpoint blockers, I asked whether DTA-1 take parts in exhaustion state of tumor associated CD8 T cells. To see the exhaustion status, I examined the inhibitory receptors such as TIM-3, PD-1, 2B4 and LAG3 from CD8 T cells in tumor site from DTA-1 treated mice. Surprisingly, DTA-1 induced inhibitory receptor expression from tumor infiltrated CD8 T cells **(Figure10)**. On the other hand, effector molecule production is also increased by DTA-1 **(Figure11)**. From these results, I suggest that DTA-1 induced inhibitory receptors are early activation marker of tumor infiltrating CD8 T cells and DTA-1 is not much associated with exhaustion.

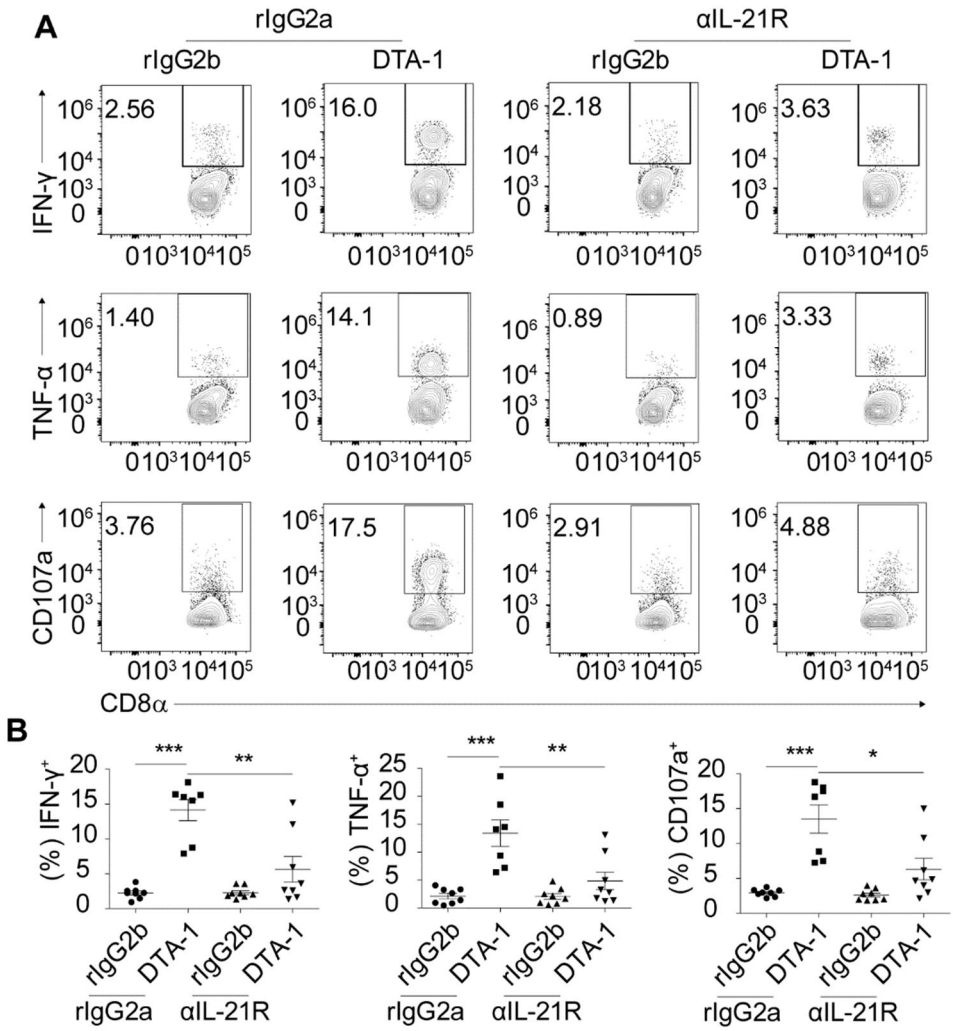

Figure 9.7 DTA-1 induced IL-21 mediates effector molecule production by CD8 T cells.

CT26 tumor bearing mice were treated with DTA-1 or αIL-21R antibodies. 8 days after DTA-1 treatment, spleen and tumor draining lymph node (TdLN) cells were harvested and stimulated with CT26 epitope AH-1 for 5 days. After 5 days stimulation, cells were re-stimulated with AH-1 for 4 hours followed by intracellular staining of IFN-ɣ, TNF-α and CD107a and analyzed in CD8 T cells. Representative dot plots **(A)** and graph data **(B)** of effector molecule production from CD8 T cells are depicted. **(A and B)** The data represent at least two experiments. **(B)** Results were shown as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P< 0.005$ as determined using two way ANOVA.

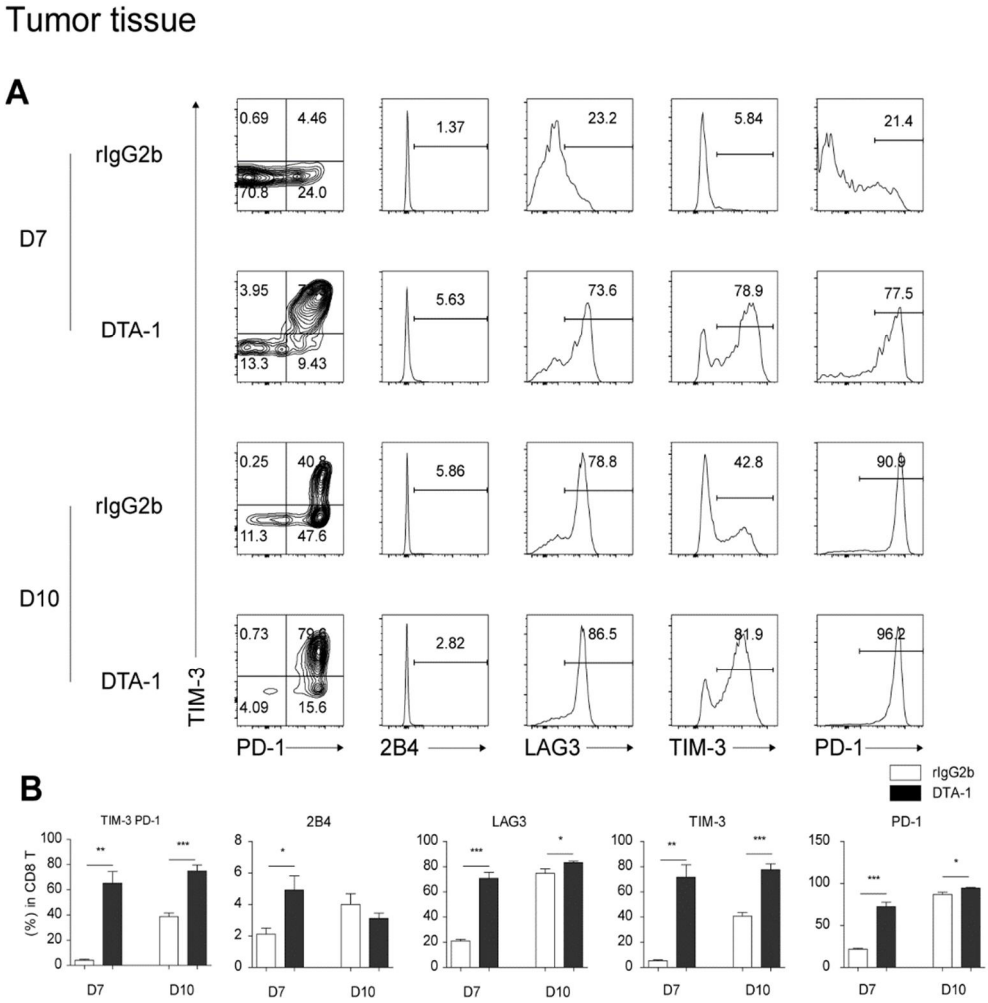

Figure 10. Inhibitory receptor expressions from TIL CD8 T cells.

7 and 10 days after DTA-1 treatment, immune cells harvested from tumor tissue of each group are followed by staining of inhibitory receptors including TIM-3, PD-1, 2B4 and LAG-3 are analyzed in CD8 T cells.

Representative dot plots **(A)** and graph data **(B)** of each receptor expressions are depicted. **(A and B)** The data represent at least two experiments. **(B)** Results were shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using two way ANOVA.

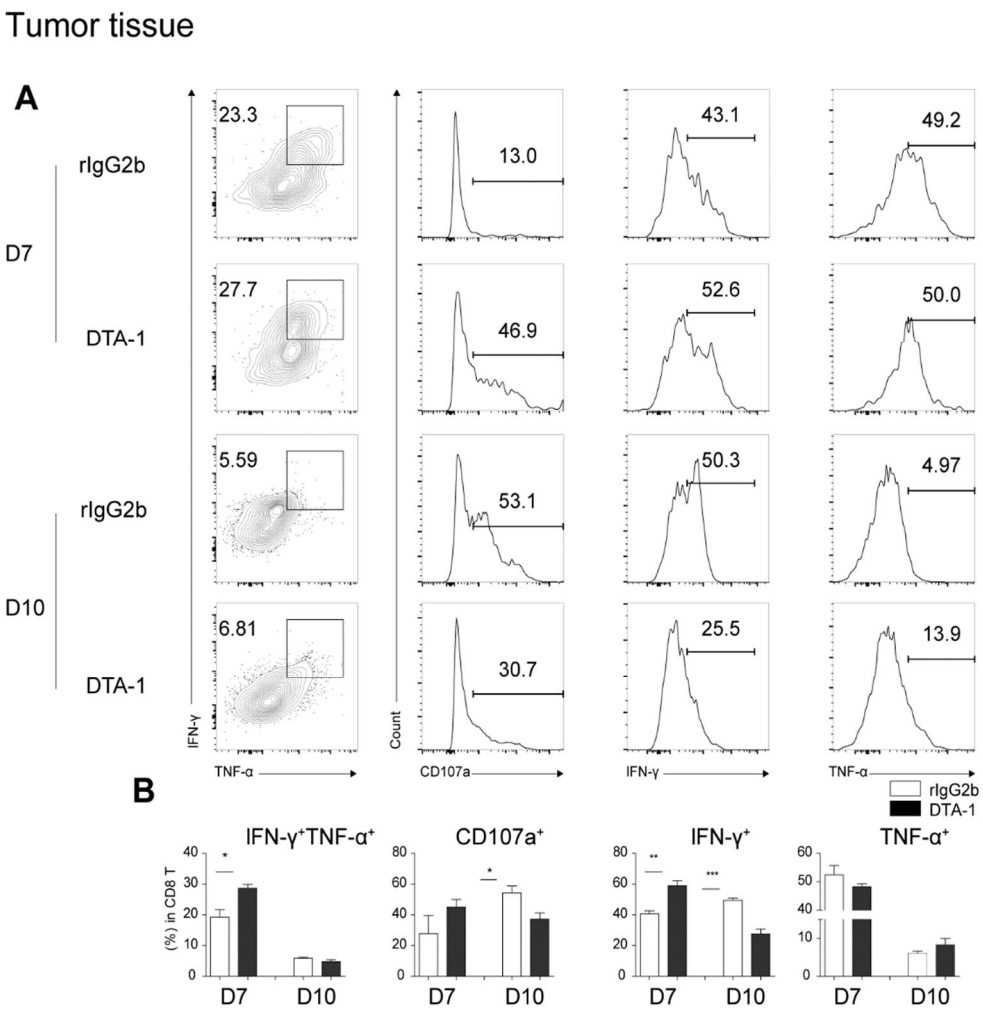

Figure 11. Effector molecule productions from TIL CD8 T cells.

7 and 10 days after DTA-1 treatment, immune cells harvested from tumor tissue of each group are stimulated with PMA, ionomycin for 4 hours and followed by staining of IFN-ɣ, TNF-α and CD107a and analyzed in CD8 T cells. Representative dot plots **(A)** and graph data **(B)** of each molecule expressions are depicted. **(A and B)** The data represent at least two experiments. **(B)** Results were shown as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P< 0.005$ as determined using two way ANOVA.

In addition to its impact on effector cells, IL-21 is also known as a negative regulator of suppressive Treg cells, by affecting differentiation and survival (51,52). I also previously reported that DTA-1 hampers the differentiation of naïve CD4 T cells into Treg cells which resulted in reduced Treg populations in tumor tissue (20). To examine whether DTA-1 induced Treg cell decrement is IL-21-dependent, I analyzed the $Foxp3^+$ tumor infiltrating lymphocyte (TIL) Treg cell population in tumor bearing mice treated with DTA-1 and αIL-21R and found that blocking IL-21 signaling had no effect on the TIL Treg population **(Figure 12)**.

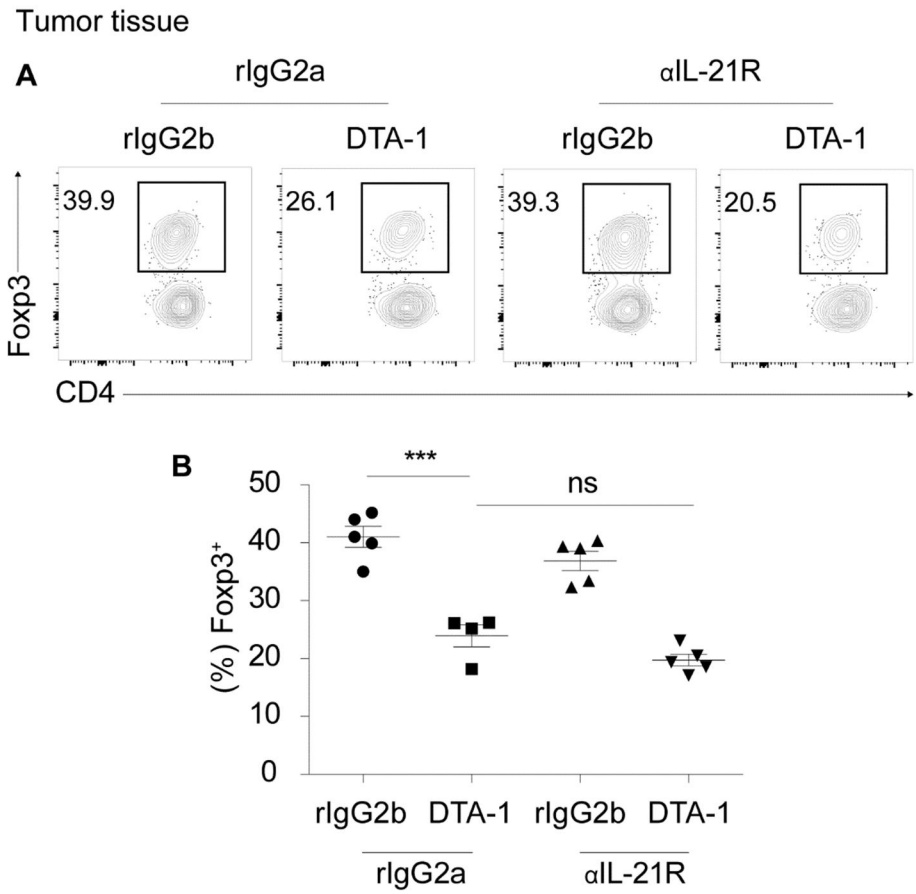

Figure 12. Population of Treg cells are not affected by IL-21 inhibition.

8 days after DTA-1 treatment, population of FoxP3$^+$ Treg cells are analyzed in tumor tissues from each group. Representative dot plots **(A)** and graph data **(B)** of Treg cells are shown. **(A and B)** The data represent at least two experiments. **(B)** Results were shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using two way ANOVA.

Collectively, these results demonstrate that DTA-1-induced IL-21 facilitates the antitumor immune responses by activating CD8 T cells.

Tfh cells mainly produce IL-21 upon DTA-1 treatment in tumor-bearing mice.

As previously described (20), treatment of DTA-1 increased IL-21 expression in the CD4 T cells of tumor-draining lymph node (TdLN) at the transcript level **(Figure 13A)**. Next, I examined the kinetics of IL-21 expression in CD4 T cells followed by DTA-1 treatment and found that the IL-21 expression in CD4 T cells was significantly increased at day 4 and peaked at day 7 and then dramatically decreased thereafter **(Figure 13B)**.

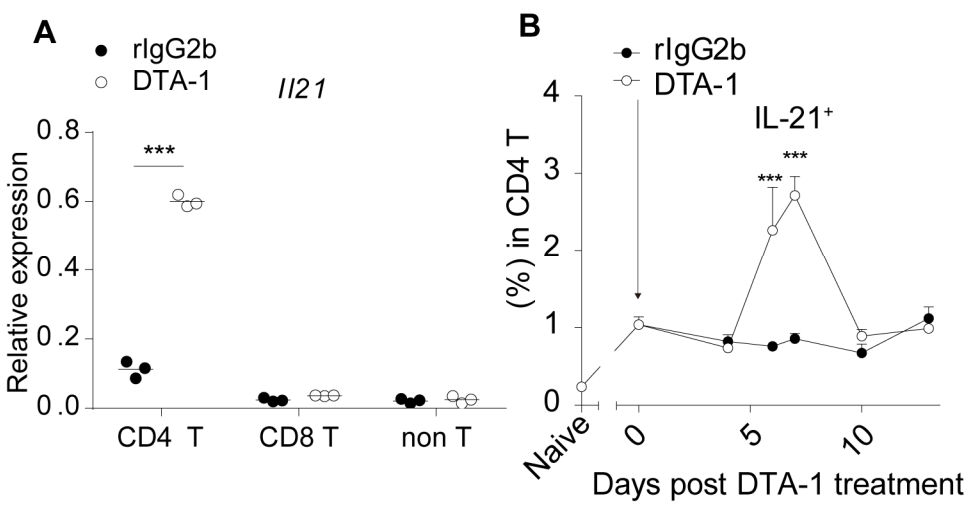

Figure 13. DTA-1 increases IL-21 production from CD4 T cells.

(A and B) Balb/c mice were subcutaneously injected CT26 tumor cells. 5 days after tumor inoculation, mice were intraperitoneally injected with DTA-1 or control rIgG2b antibodies. **(A)** 6 days after DTA-1 treatment, *Il21* mRNA transcript expression of CD4 T, CD8 T and non T cells from TdLN of tumor bearing mouse were qPCR analyzed. **(B)** Kinetic analysis of the percentage of IL-21 secreting CD4 T cells from TdLN were shown. **(A and B)** The data represent at least two experiments. **(A)** Results were shown as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P< 0.005$ as determined using a Student's t-test. **(B)** Results were shown as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P< 0.005$ as determined using two way ANOVA.

To determine the structural changes induced by DTA-1 in TdLN at day 7, I performed IHC staining. The immunohistological analysis showed that treatment with DTA-1 enhanced germinal center responses in vivo **(Figure 14)**.

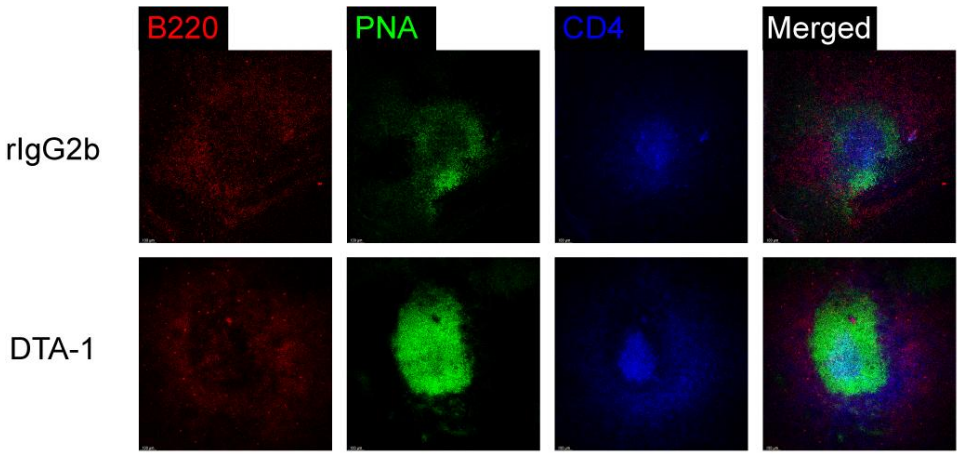

Figure 14. Immunohistology of tumor draining lymph nodes in DTA-1 treated mice

CT26 tumor bearing mice were treated with DTA-1 or rIgG2b control. 7 days after DTA-1 treatment, tumor draining lymph nodes sections were stained with anti-B220, anti-PNA and anti-CD4 antibodies. The data represent two independent experiments.

Given that Tfh cells which mediate germinal center responses are one of the well-known cellular sources of IL-21, I investigated whether DTA-1-induced IL-21 production is found in Tfh cells. As I expected, DTA-1 treatment increased the frequency of IL-21–producing cells and almost 80%

of these cells were coexpressing CXCR5 and PD-1, which are typical surface markers for Tfh cells and the number of $CXCR5^{+}PD\text{-}1^{+}$ cells also peaked at 7 days after DTA-1 treatment as did the $IL\text{-}21^{+}$ CD4 T cells **(Figure 15A and B)**. In addition to frequency, the absolute number of IL-21–expressing–$CXCR5^{+}$ $PD\text{-}1^{+}$ cells was also significantly increased in DTA-1-treated mice compared to rIgG2b-treated mice **(Figure. 15C)**.

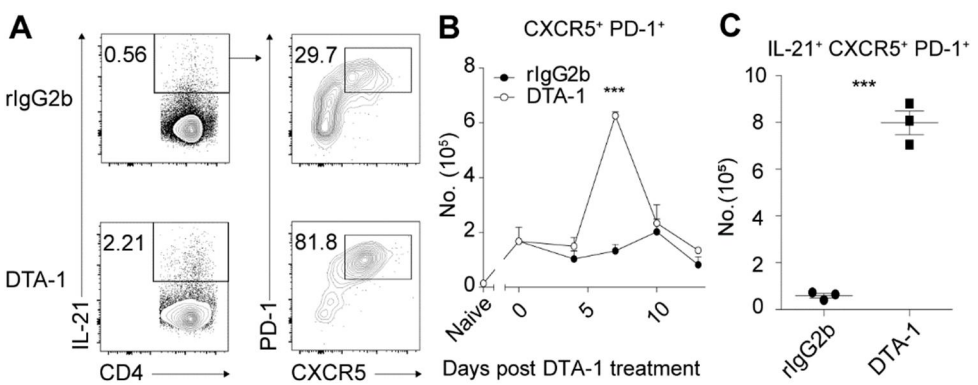

Figure 15. DTA-1 induced IL-21 producing CD4 T cells express CXCR5 and PD-1.

(A) CXCR5 and PD-1 expression in IL-21 producing CD4 T cells from TdLN of DTA-1 treated CT26 tumor bearing mouse. **(B)** Kinetic analysis of CXCR5 and PD-1 expressing cells from TdLN after DTA-1 treatment. **(C)** Numbers of $IL\text{-}21^{+}$ $CXCR5^{+}$ $PD\text{-}1^{+}$ CD4 T cells are described in graph. **(A,**

B and C) The data represent at least two experiments. **(B)** Results were shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using two way ANOVA. **(C)** Results were shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using Student's t-test.

Coculture experiments revealed that DTA-1-induced $CXCR5^{+}PD\text{-}1^{+}$ CD4 T cells were genuine Tfh cells as they increased IgG production from B cells *in vitro* **(Figure 16)**.

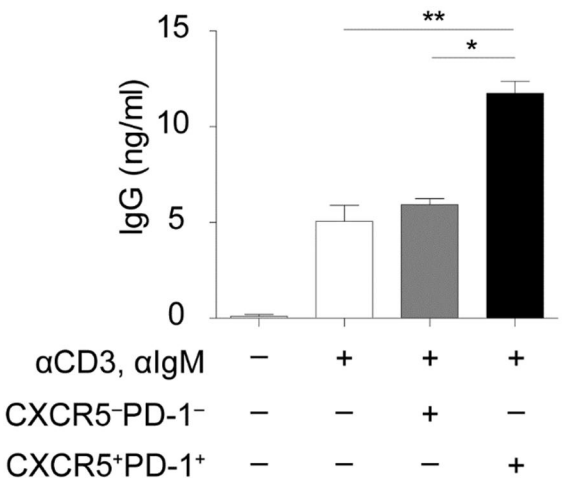

Figure 16. DTA-1 induced $CXCR5^{+}PD\text{-}1^{+}$ CD4 T cells stimulate B cells *in vitro*.

CT26 cells were s.c. injected into BALB/c mice, which were treated with DTA-1 7 days after tumor inoculation. After another 7 days later, $CXCR5^{-}$ $PD\text{-}1^{-}$, $CXCR5^{+}PD\text{-}1^{+}$ CD4 T cells were harvested from TdLNs and cocultured with B cells from TdLNs of Ab-free mice in the presence of αCD3 and αIgM. Concentration of IgG in the accumulated supernatant collected from 6-day cocultured wells with indicated conditions. The data represent two independent experiments. Results are shown as the mean ± SEM. * $P<0.05$, ** $P<0.01$, and *** $P< 0.005$ as determined using Student's t-test.

I also observed that DTA-1 treatment potently induced IL-21–expressing $CXCR5^{+}PD\text{-}1^{+}$ Tfh cells in other tumor models such as B16F10, MC38, and TC-1 cells **(Figure 17)**.

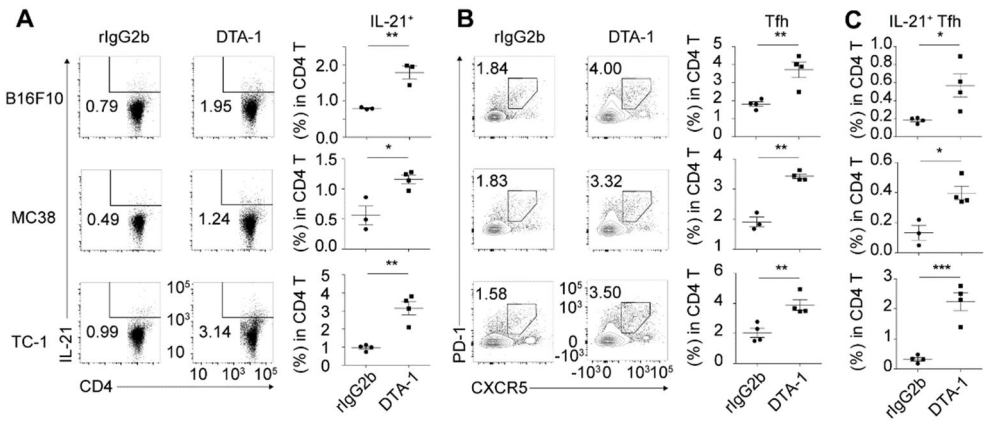

Figure 17. DTA-1 induces IL-21$^+$ Tfh cells in various tumor models

C57BL/6 mice were s.c. injected with each tumor cell line; B16F10, MC38 or TC-1. Five days after tumor inoculation, the mice were treated with DTA-1 or control rIgG2b antibodies and TdLNs were analyzed 7 days after DTA-1 administration. The populations of IL-21$^+$ cells **(A)**, CXCR5$^+$PD-1$^+$ Tfh cells **(B)** and IL-21$^+$CXCR5$^+$PD-1$^+$ Tfh cells **(C)** in CD4 T cells from TdLNs are shown as dot plots and the bar graphs. The data represent two independent experiments and results are shown as the mean ± SEM. * P<0.05, ** P<0.01, and *** P< 0.005 as determined using Student's t-test.

I further analyzed tumor-infiltrating lymphocytes and found that IL-21–producing Tfh cells were also increased by DTA-1 treatment in tumor tissue

(Figure 18).

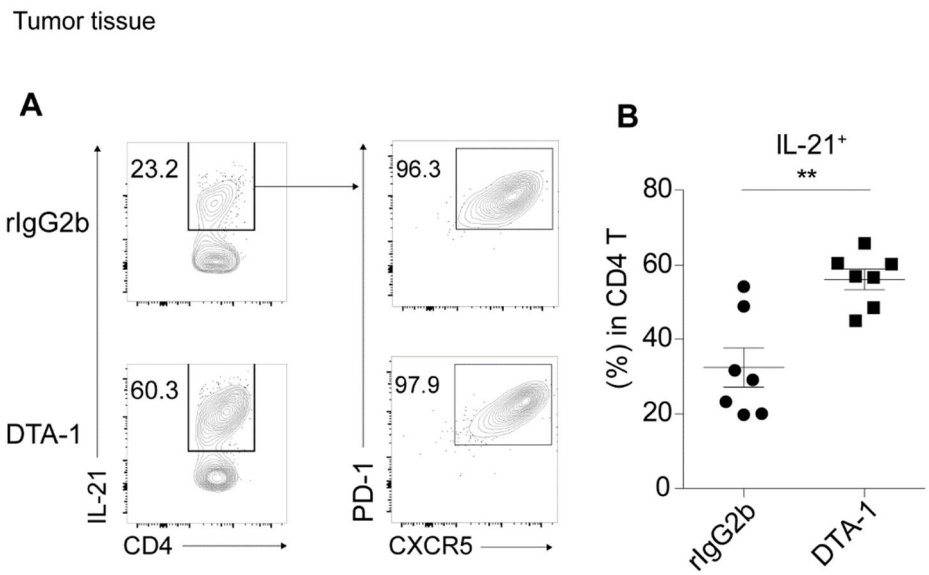

Figure 18. DTA-1 increases frequency of IL-21$^+$ Tfh cells in tumor tissue

Tumor infiltrating lymphocytes were harvested and followed by staining with IL-21, CXCR5 and PD-1 in CD4 T cells. Dot plots of IL-21$^+$ Th cells (left) and CXCR5 and PD-1 expression in IL-21$^+$ Th cells (right) **(A)** and the frequency of IL-21$^+$ CD4 T cells **(B)** in tumor tissue were shown. **(A and B)**The data represent at least two experiments and **(B)** results were shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using a Student's t-test.

Taken together, I found that DTA-1-induced IL-21 is mainly produced by Tfh cells.

Antigen specific induction of IL-21–producing Tfh cells by DTA-1.

Next, I asked whether the induction of IL-21–expressing Tfh cells by DTA-1 treatment is antigen-specific. I first compared the frequencies of IL-21–expressing Tfh cells in the distal lymph node (distal LN), which is the opposite side from tumor injected flank, and tumor draining lymph node (TdLN). I found that IL-21–producing CD4 T cells and Tfh cells were only increased in TdLNs after DTA-1 treatment **(Figure 19)**.

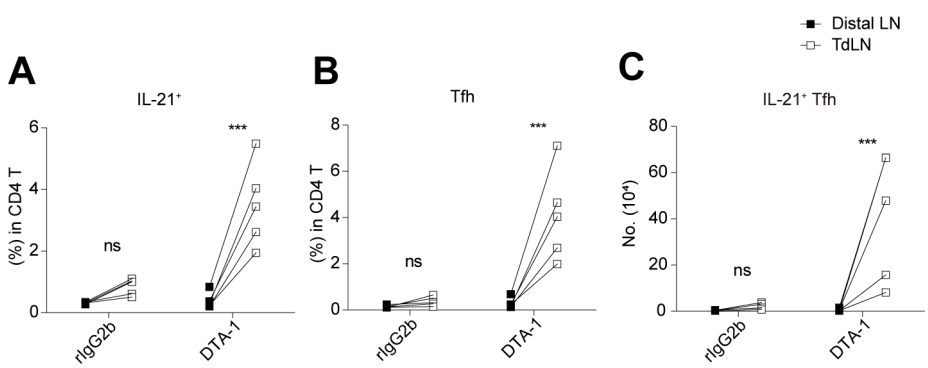

Figure 19. DTA-1 elevates population of IL-21$^+$ Tfh cells only in draining lymph nodes

7 days after DTA-1 treatment, the percentage of IL-21$^+$ CD4 T cells **(A),** Tfh cells **(B)** and IL-21$^+$ Tfh cells **(C)** from ndLN and TdLN are depicted in graph. **(A, B and C)** The data represent at least two experiments and results were shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using a Student's t-test.

To directly determine the antigen-specificity, I subcutaneously injected mice with B16F10-OVA tumor cells and then adoptively transferred a 1:1 mixture of CD45.1$^+$CD45.2$^+$ OVA-specific CD4 T cells and CD45.1$^+$CD45.2$^-$ polyclonal CD4 T cells 9 days after tumor inoculation. Compared to the CD45.1$^+$CD45.2$^-$ polyclonal CD4 T cells, the IL-21–secreting CXCR5$^+$PD-1$^+$ Tfh cells were significantly increased in CD45.1$^+$CD45.2$^+$ OVA specific CD4 T cells upon DTA-1 treatment **(Figure 20)**.

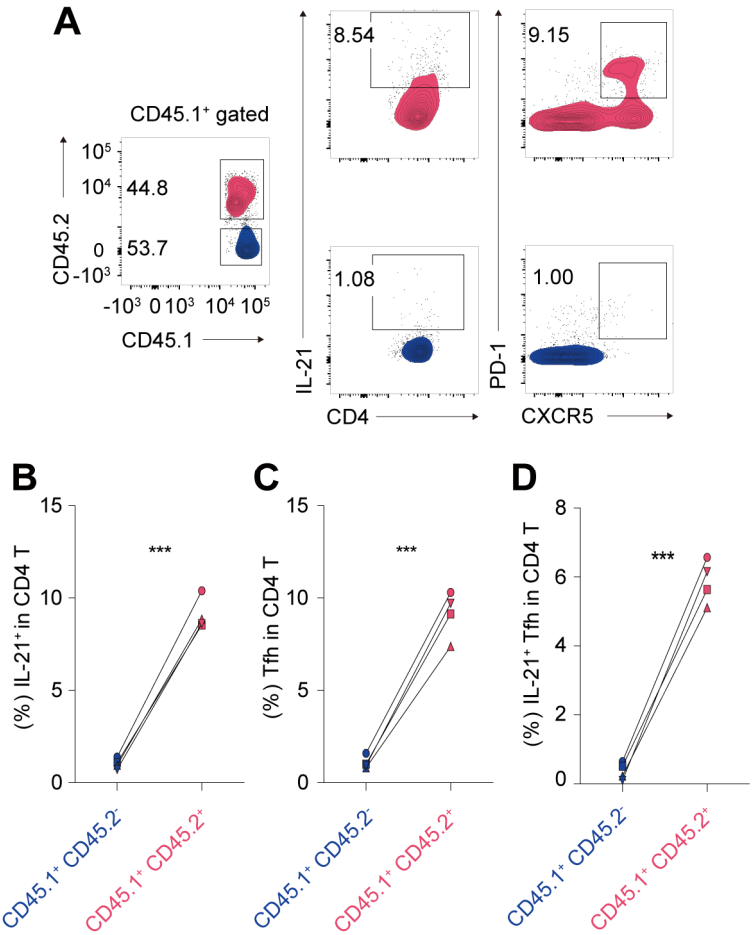

Figure 20. IL-21$^+$ Tfh cells are induced by DTA-1 in antigen specific manner

C57BL/6 mouse were subcutaneously injected with 10^6 B16F10-OVA(MO5) cells. 9 days after tumor cell injection, CD4 T cells from CD45.1$^+$ mouse and CD45.1$^+$ CD45.2$^+$ OT-II mouse were adoptively transferred into tumor bearing mouse. Next day, DTA-1 and control rIgG2b antibodies were i.p.

treated. TdLN were harvested and analyzed 7 days after antibodies treatment. **(A)** Contour plots show the IL-21, CXCR5 and PD-1 expression from $CD45.1^+$ cells. Red plots show $CD45.1^+$ $CD45.2^+$ OT-II cells and blue plots show $CD45.1^+$ cells. The percentage of $IL\text{-}21^+$ cells **(B)**, Tfh cells **(C)** and $IL\text{-}21^+$ Tfh cells **(D)** are depicted in graph data. **(A - D)** The data represent at least two experiments and **(B, C and D)** results were shown as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P< 0.005$ as determined using a Student's t-test.

Taken together, these data suggest that the induction of $IL\text{-}21^+$ Tfh cell populations by GITR stimulation is an antigen-specific process.

IL-4 is required for the DTA-1 induced-IL-21–producing Tfh cells.

Next, I investigated the mechanism by which DTA-1 induces IL-21 production in CD4 T cells. First, I examined whether DTA-1 stimulation directly induces IL-21 production from CD4 T cells *in vitro*. I found that DTA-1 induced IL-21 production from total CD4 T cells **(Figure 21)**.

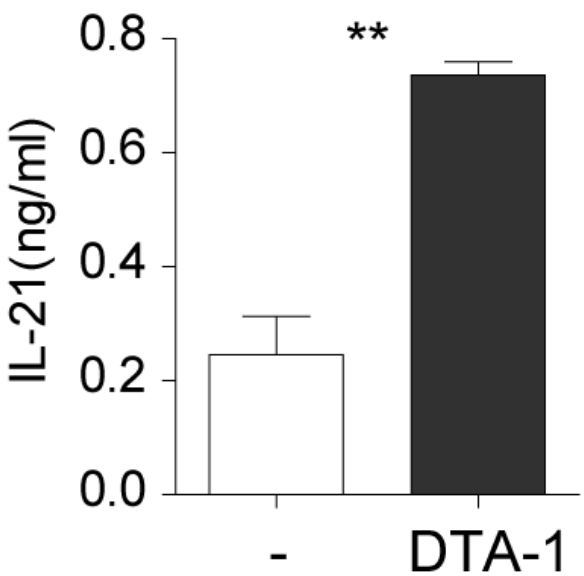

Figure 21. DTA-1 stimulation directly induces IL-21 production from total CD4 T cells

Total CD4 T cells were harvested isolated from pooled spleen and lymph

nodes of naïve BALB/c mice and stimulated with αCD3 and αCD28 in the presence of DTA-1. Concentration of IL-21 from 4 days accumulated supernatant were determined by ELISA. The data represent at least two independent experiments. Results are shown as the mean ± SEM. * $P<0.05$, ** $P<0.01$, and *** $P< 0.005$ as determined using Student's t-test.

To determine whether IL-21 is produced from effector or naïve CD4 T cells, I observed IL-21 production with different initial ratios of naïve and effector CD4 T cells. Of interest, neither naïve nor effector derived CD4 T cells alone produced IL-21 in response to DTA-1 stimulation, whereas the combination of naïve and effector CD4 T cells synergistically produced a substantial amount of IL-21 **(Figure 22)**.

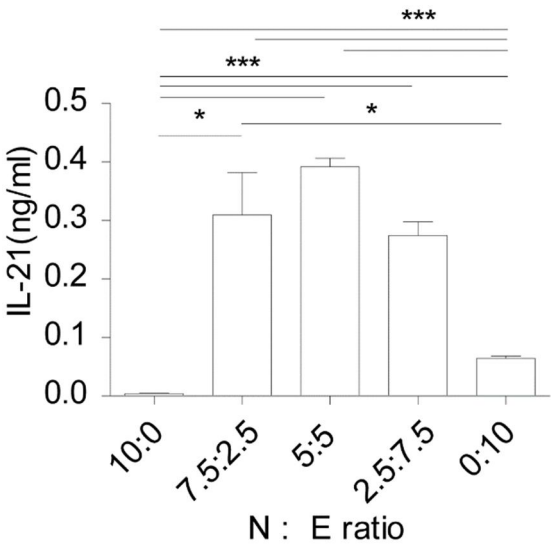

Figure 22. Both naïve and effector CD4 T cells are required for optimal IL-21 production by DTA-1 stimulation

$CD4^+CD25^-CD44^-CD62L^+$ Naïve (N) and $CD4^+CD25^-CD44^+CD62L^-$ effector (E) CD4 T cells were *in vitro* cultured for 4 days as indicated ratio with DTA-1. IL-21 production from each cell ratio are depicted in graphical display. The data represent two independent experiments and results were shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using a Student's t-test.

To clarify the source of IL-21, I cocultured $Thy1.1^+CD44^+CD62L^-$ cells and $Thy1.2^+CD44^-CD62L^+$ cells stimulated with αCD3 and αCD28 in the

presence of DTA-1. I found that IL-21 was produced by only restimulated Thy1.2^{+} CD4 T cells that were derived from CD44^{-}CD62L^{+} naïve CD4 T cells **(Figure 23)**. Taken together, DTA-1 stimulation differentiated naïve CD4 T cells into IL-21–producing CD4 T cells.

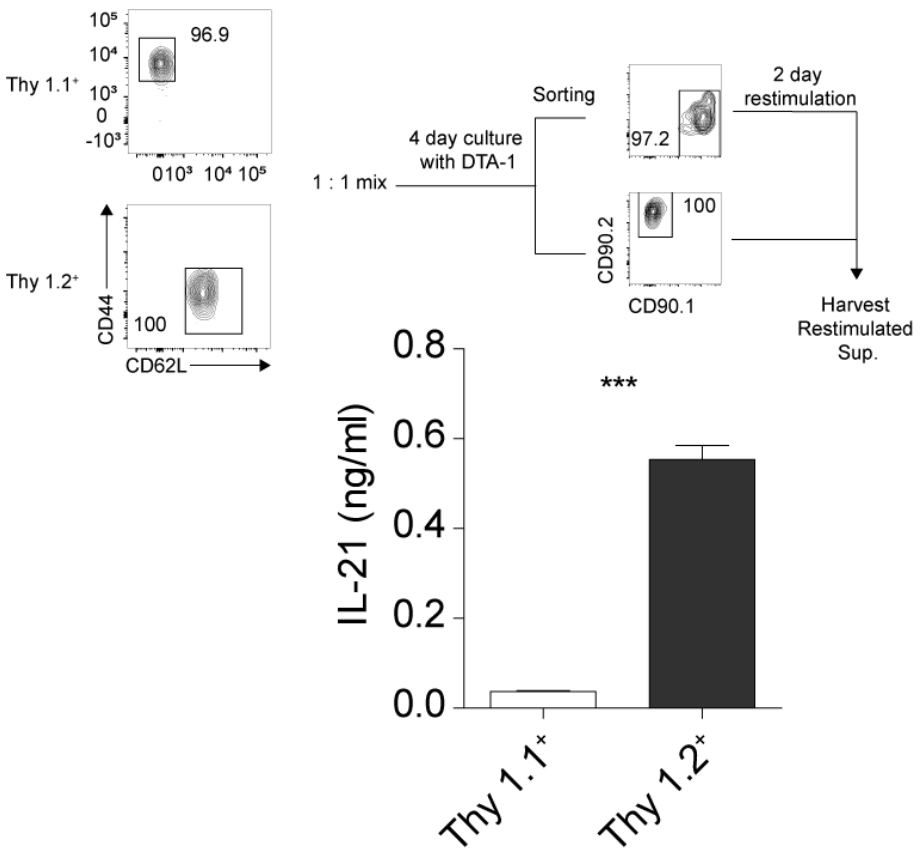

Figure 23. DTA-1 induced IL-21 is produced by naïve derived CD4 T cells

$CD4^+CD25^-CD44^+CD62L^-$ effector CD4 T cells were harvested from Thy 1.1^+ mice and $CD4^+CD25^-CD44^-CD62L^+$ naïve CD4 T cells were harvested from Thy 1.2^+ mice. 1 : 1 mixture of these cells were stimulated with αCD3 and αCD28 in the presence of DTA-1. 4 days after stimulation, Thy 1.1^+ cells and Thy 1.2^+ cells were FACS sorted and stimulated with αCD3 and αCD28 respectively. IL-21 concentration from restiumlated supernatant were determined by ELISA. The data represent at least two independent experiments. Results are shown as the mean ± SEM. * $P<0.05$, ** $P<0.01$, and *** $P< 0.005$ as determined using Student's t-test.

The differentiation of naïve CD4 T cells into each Th subset is dependent on cytokine stimulation. Thus, I examined cytokines related to IL-21 production in the TdLNs upon DTA-1 treatment. I found that IL-4, -6, and -27 but not IL-12 were increased by DTA-1 treatment (Figure 24).

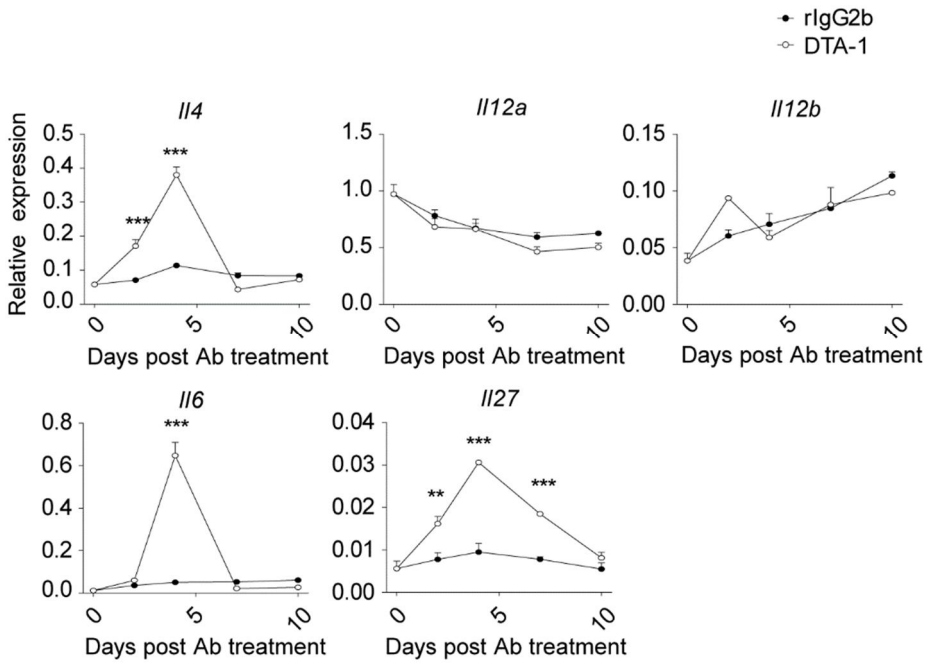

Figure 24. Kinetic analysis of IL-21 associated cytokines

mRNA transcripts of *Il4, Il12a, Il12b, Il6 and Il27* are determined after DTA-1 treatment. The data represent two experiments and results were shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using two way ANOVA.

For mechanistic studies, I analyzed IL-21 production from CD4 T cells upon DTA-1 stimulation *in vitro*. Neutralizing antibodies to each cytokine showed that the IL-21 production increased by DTA-1 stimulation was only reversed

by adding antibodies against IL-4 **(Figure 25)**.

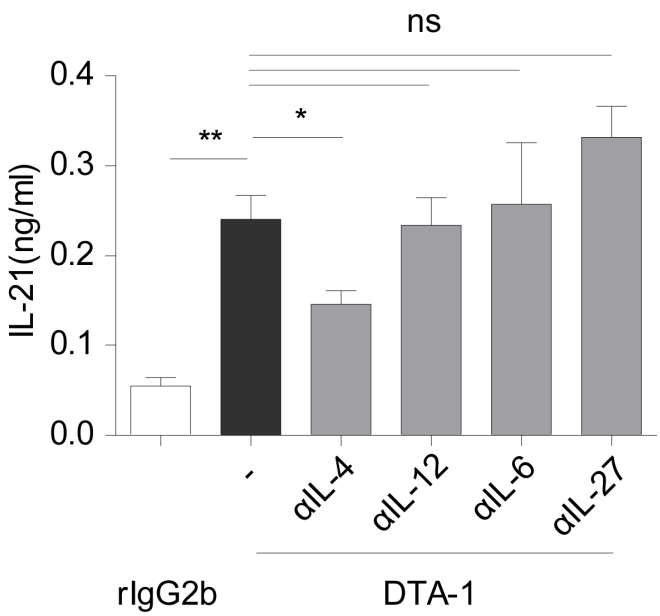

Figure 25. IL-4 is required for IL-21 production from GITR costimulated CD4 T cells *in vitro*

IL-21 concentration from accumulated supernatant of *in vitro* cultured CD4 T cells stimulated with DTA-1 or control rIgG2b for 4 days with indicated cytokine neutralizing antibodies. The data represent two independent experiments and results were shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using a Student's t-test.

Furthermore, while DTA-1 stimulation did not increase IL-21 production in naïve CD4 T cells during neutral or other cytokine conditions, it dramatically increased IL-21 production in the presence of IL-4 **(Figure 26)**.

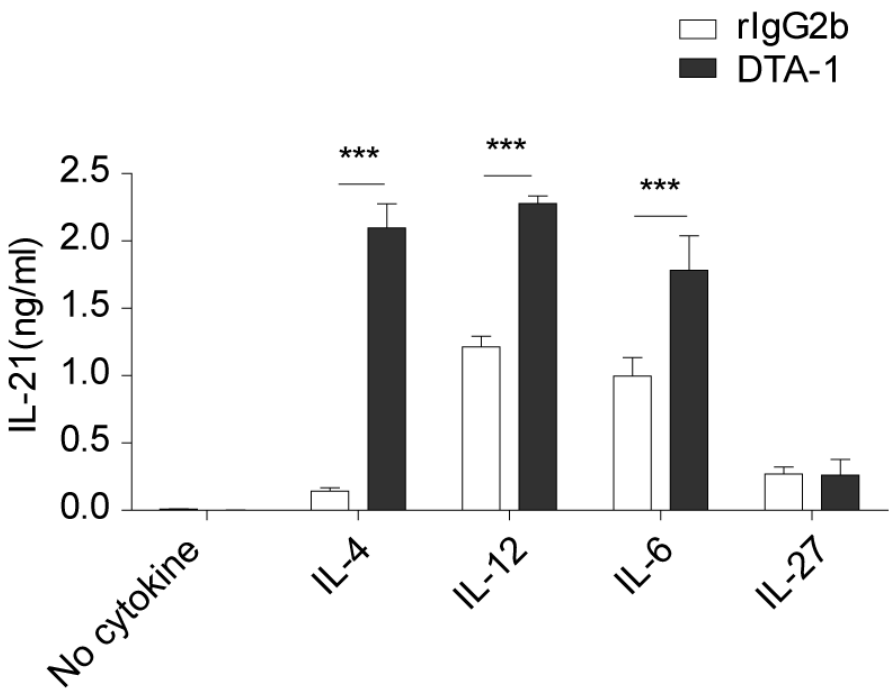

Figure 26. GITR stimulation significantly increases IL-21 production from naïve CD4 T cells with IL-4

Concentration of IL-21 from accumulated supernatant of *in vitro* cultured naïve CD4 T cells stimulated with DTA-1 or control rIgG2b with indicated cytokines. The data represent two independent experiments and results were

shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using a Student's t-test.

Moreover, GITR ligation also promoted IL-21 production in human naïve CD4 T cells in the presence of IL-4 **(Figure 27)**, suggesting the important role of IL-4 in GITR-induced IL-21 production.

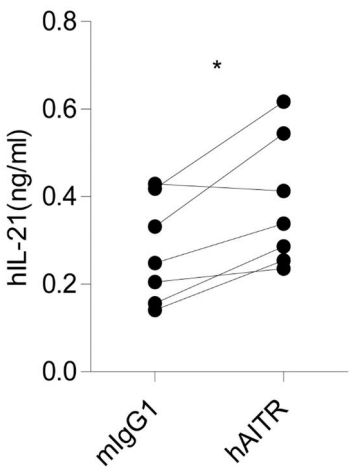

Figure 27. Human AITR increases IL-21 production from human naïve CD4 T cells with IL-4

IL-21 production by human naïve CD4 T cells stimulated with hAITR or control mIgG1 under IL-4 condition. The data represent two independent

experiments and results were shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using a Student's t-test.

As I demonstrated that both naïve and effector CD4 T cells are required for DTA-1 induced IL-21 production *in vitro* **(Figure 22)**, I hypothesized that DTA-1 induced IL-21 is produced by naïve derived CD4 T cells stimulated with DTA-1 and IL-4 which is produced by effector CD4 T cells. Thus, I examined IL-21 production from CD4 T cells comprised of IL-4 receptor-deficient naïve or effector CD4 T cells. DTA-1 stimulation on CD4 T cells produced IL-21 with IL-4 receptor deficiency in effector CD4 T cells. However, IL-4 receptor deficiency in naïve CD4 T cells resulted in significantly reduced IL-21 production by DTA-1 stimulation *in vitro* **(Figure 28)**.

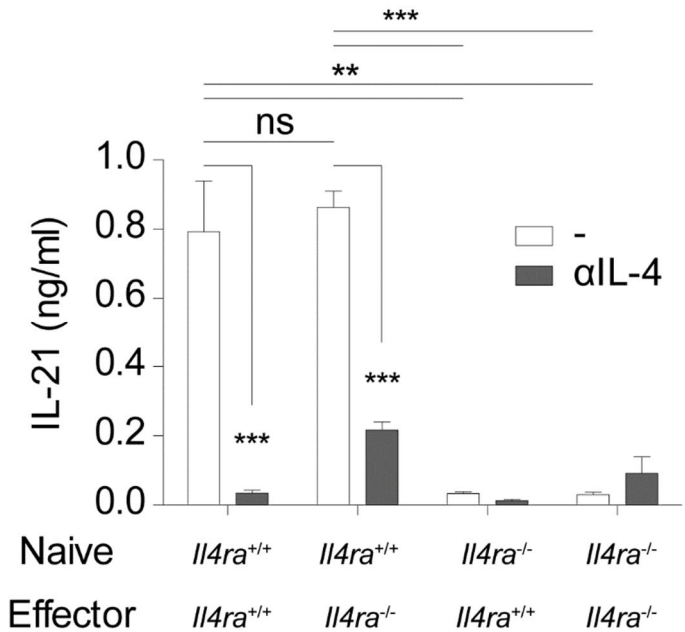

Figure 28. IL-4 signal transduction into naïve CD4 T cells is required for IL-21 production upon GITR stimulation *in vitro*.

1 : 1 mixture of $CD4^+CD25^-CD44^-CD62L^+$ naïve and $CD4^+CD25^-CD44^+CD62L^-$ effector CD4 T cells from *Il4ra*$^{+/+}$ (WT) or *Il4ra*$^{-/-}$ (KO) mice were cultured for 4 days as the indicated combination with 2μg/ml DTA-1 *in vitro*. The IL-21 production is depicted in the graphical display and grey bar represent the wells added with 2μg/ml of αIL-4 neutralizing antibodies. The data represent two independent experiments and results are shown as the mean ± SEM. * $P<0.05$, ** $P<0.01$, and *** $P< 0.005$ as determined using two way ANOVA and Student's t-test.

Using IL-4 receptor-deficient mice, I found that the increased frequencies of IL-21–producing CD4 T cells as well as Tfh cells upon DTA-1 treatment were significantly reduced in IL-4 receptor-deficient mice compared to littermate controls *in vivo* **(Figure 29)**. Altogether, these results suggest that IL-4 is essential for IL-21 upregulation by CD4 T cells when GITR costimulation is supplemented.

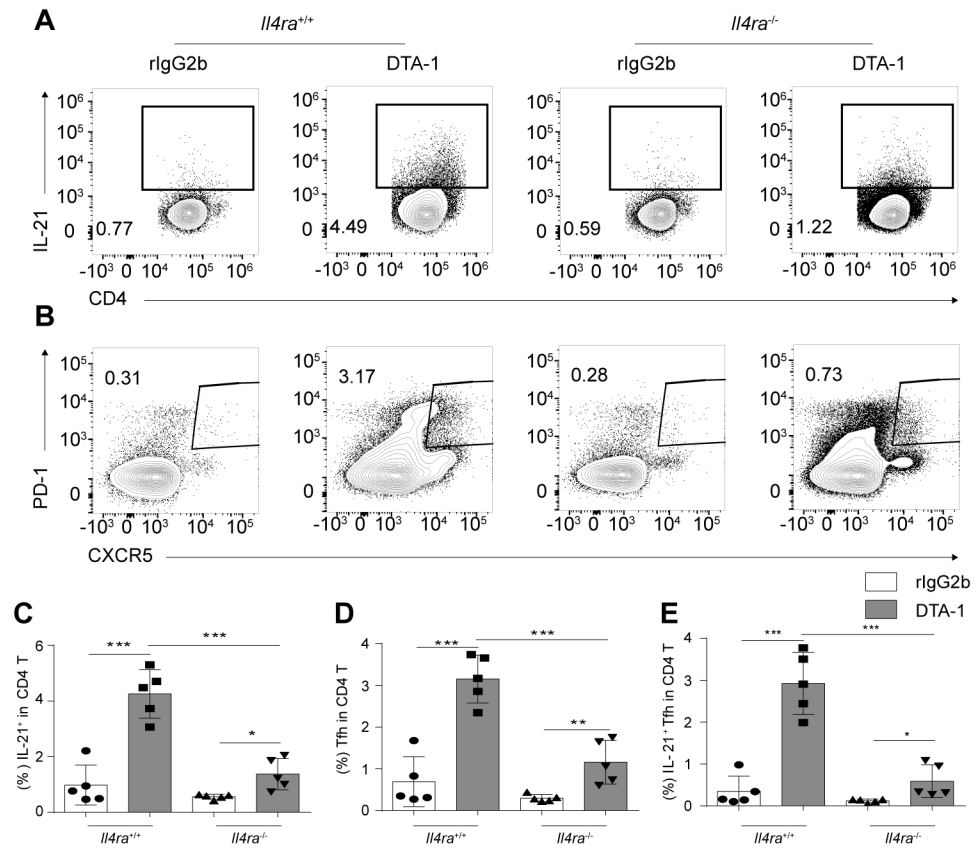

Figure 29. IL-4 signal is required for IL-21$^+$ Tfh cell induction *in vivo*.

CT26 tumor bearing *Il4ra* knockout mice are treated with DTA-1. 7 days later, population of IL-21$^+$ CD4 T cells **(A and C)**, Tfh cells **(B and D)** and IL-21$^+$ Tfh cells **(E)** are depicted. **(A - E)** The data represent at least two experiments. **(C – E)** Results were shown as mean ± SEM. * P<0.05, ** P<0.01, *** P< 0.005 as determined using a Student's t-test.

IL-21 induction by DTA-1 is independent from IL-9

Given that DTA-1-induced IL-9 mediates antitumor immune responses and that DTA-1-induced IL-9 expression preceded IL-21 expression, I tested whether the IL-21 induction by DTA-1 is dependent on IL-9. I found that neutralization of IL-9 did not alter the frequencies of IL-21–expressing CD4 T cells or Tfh cells in DTA-1-treated mice **(Figure 30A and B)**. In line with this finding, IL-9 did not induce IL-21 production in naïve CD4 T cells *in vitro* **(Figure 30C)**.

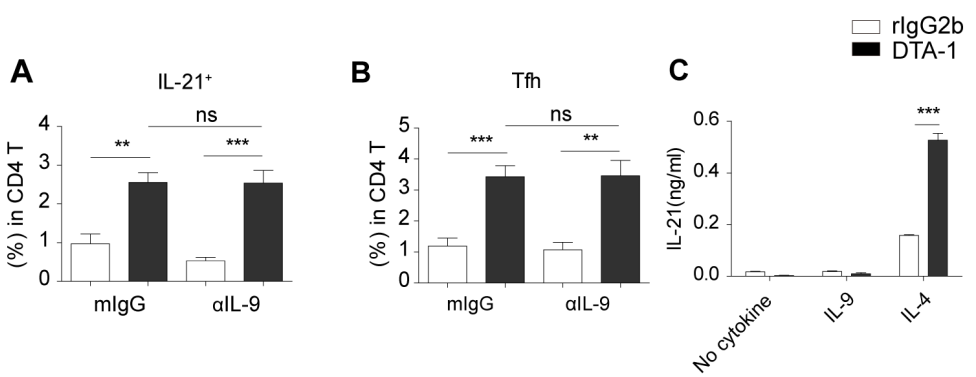

Figure 30. IL-9 is independent from IL-21 production and Tfh population induction by DTA-1.

(A and B) CT26 tumor bearing mice were treated with DTA-1 5 days after

tumor challenge and IL-9 neutralizing antibodies were treated 7, 9 and 11 days after tumor injection. The percentage of IL-21^{+} Th cells **(A)** and Tfh population **(B)** in CD4 T cells are shown. **(C)** Concentration of IL-21 from accumulated supernatant of IL-4 or IL-9 and DTA-1 conditioned naïve CD4 T cells *in vitro*. **(A, B and C)** The data represent at least two experiments and results were shown as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P< 0.005$ as determined using a Student's t-test.

Blocking both IL-9 and IL-21 signaling made any significant difference in tumor growth from blocking each **(Figure 31)**. Taken together, DTA-1 induced IL-21 production is independent of IL-9.

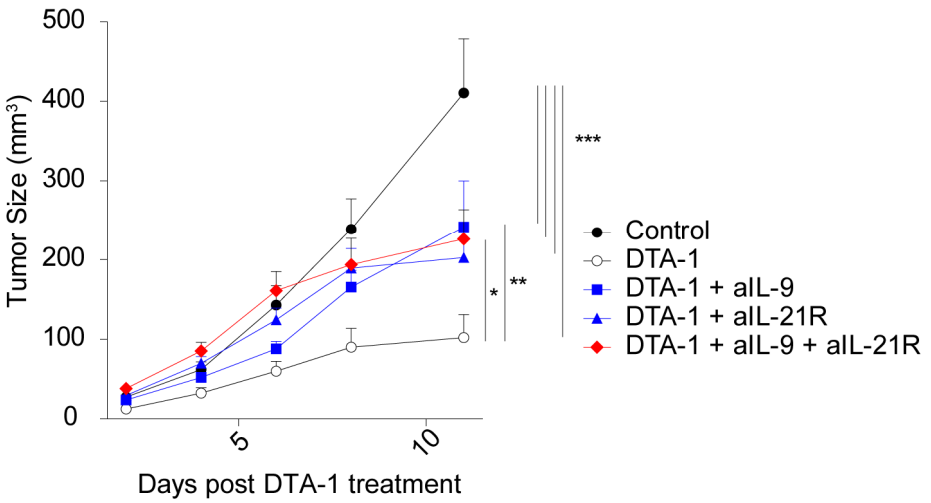

Figure 31. Tumor size from DTA-1, αIL-9 and αIL-21R treated mice.

CT26 tumor bearing mice were treated with DTA-1 5 days after tumor challenge followed by IL-9 neutralizing antibody treatment 7, 9 and 11 days after tumor injection and αIL-21R antibody treatment 10, 12 and 15 days after tumor injection. The data represent two experiments and results were shown as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P< 0.005$ as determined using two way ANOVA.

c-Maf mediates the development of IL-21–producing CD4 T cells by IL-4 and DTA-1.

I further dissected the molecular mechanism by which IL-4 and DTA-1 stimulation triggers IL-21 expression in CD4 T cells. To this end, I examined the mRNA expression of the transcription factors related to IL-21 expression. I found that *Maf* was markedly increased at the mRNA level by stimulation with IL-4 and DTA-1 **(Figure 32)**.

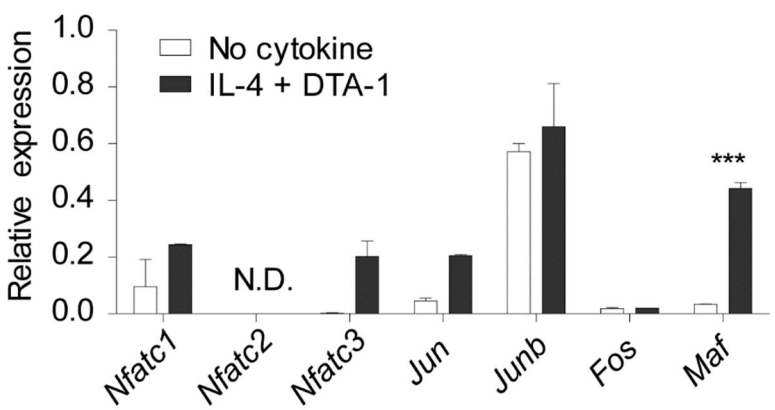

Figure 32. Expression of IL-21 associated transcription factors from naïve CD4 T cells after stimulation with IL-4 and DTA-1

mRNA transcripts from naïve CD4 T cells stimulated with IL-4 and DTA-1

for 18 hours. The data represent at least two experiments. Results are shown as the mean ± SEM. * P<0.05, ** P<0.01, and *** P< 0.005 as determined using Student's t-test.

Maf transcript expression peaked at 18 hours of stimulation and c-Maf protein was also increased after 2 days of stimulation with IL-4 and DTA-1 **(Figure 33)**.

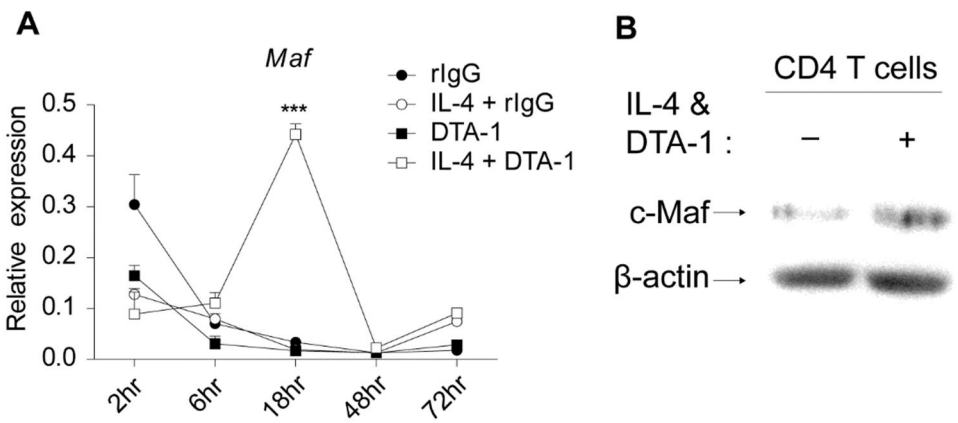

Figure 33. c-Maf is upregulated in IL-4 and DTA-1 stimulated naïve CD4 T cells

Expression of c-Maf from the CD4 T cells stimulated with IL-4 and DTA-1 at the mRNA level **(A)** and protein level after 2 days of stimulation **(B). (A and B)** The data represent at least two experiments. **(A)** The data represent

at least two experiments. Results are shown as the mean ± SEM. * P<0.05, ** P<0.01, and *** P< 0.005 as determined using two way ANOVA.

When c-Maf was downregulated using shRNA targeting c-Maf **(Figure 34A)**, the IL-21 expression in CD4 T cells was reduced compared to that of control vector-transduced T cells that were stimulated with IL-4 and DTA-1 **(Figure 34B)**. Collectively, these results suggest that the transcription factor c-Maf is important for IL-4 and DTA-1-induced IL-21 production in CD4 T cells.

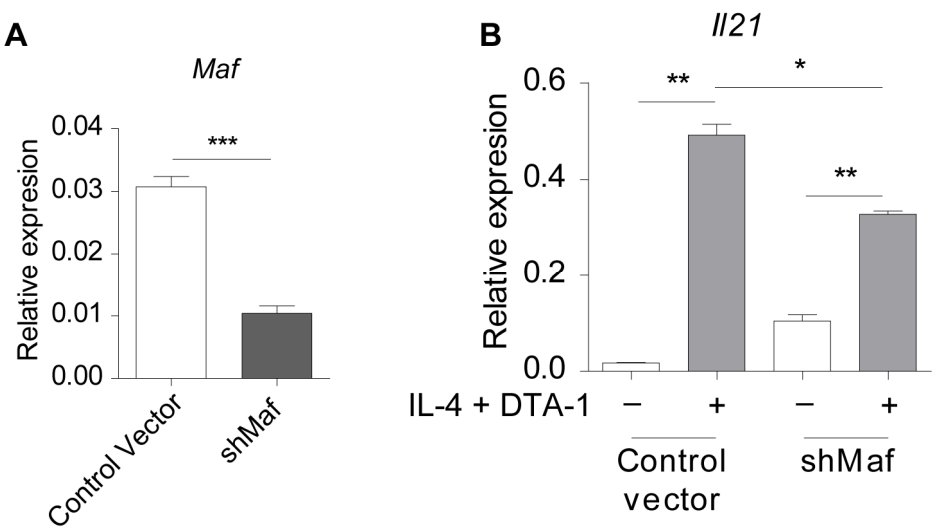

Figure 34. Downregulation of *Maf* reduces *Il21* production

(A) The *Maf* levels from CD4 T cells transduced with *Maf*–targeting shRNA(shMaf) or the control vector under IL-4 and DTA-1 conditions. **(B)** The expression of *Il21* mRNA from shMaf transduced CD4 T cells. **(A and B)** The data represent at least two experiments. Results are shown as the mean ± SEM. * P<0.05, ** P<0.01, and *** P< 0.005 as determined using Student's t-test.

Tfh cells play a critical role in the antitumor effect of DTA-1.

As I demonstrated the requirement of IL-21 in the antitumor immunity induced by DTA-1, I next asked whether Tfh cells are responsible for antitumor immunity induced by DTA-1. To address this question, I harvested Tfh cells and non-Tfh cells from B16F10-OVA tumor-bearing mice treated with DTA-1 and cocultured them with OVA-specific OT-I CD8 T cells for 2 days stimulated with T cell depleted APC and OVA peptide ($OVA_{257-264}$ and $OVA_{329-337}$). As a result, Tfh cells promoted CFSE dilution and IFN-ɣ production in responder OT-I T cells **(Figure 35)**.

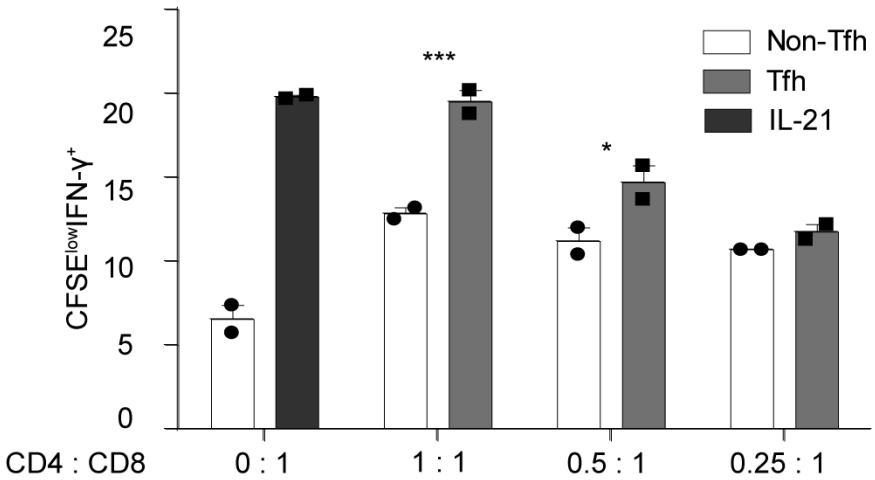

Figure 35. Tfh cells activate CD8 T cells

Tfh and non-Tfh cells from the TdLNs of B16F10-OVA tumor bearing mice treated with DTA-1 were harvested and co-cultured with 5×10^4 CFSE-labeled OT-I cells according to the indicated ratios for 2 days. Frequency of CFSE-diluted and IFN-ɣ– producing OT-I cells is shown. The data represent at least two experiments. Results are shown as the mean ± SEM. * $P<0.05$, ** $P<0.01$, and *** $P< 0.005$ as determined using Student's t-test.

To inhibit Tfh cell responses, I adopted small molecule inhibitor 79-6 which inhibits transcription factor, Bcl6, that is important for Tfh cell development and function(53,54). The Bcl6 inhibition by 79-6 decreased IL-21

production in IL-4 and DTA-1 stimulated naïve CD4 T cells **(Figure 36)** without inducing any apparent cellular cytotoxicity **(Figure 37)**.

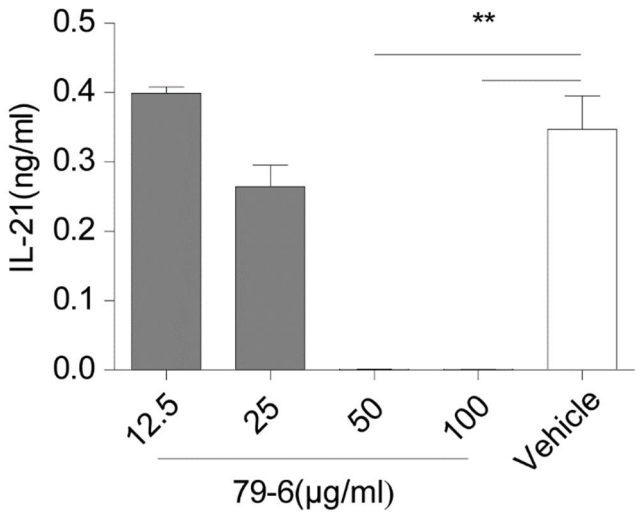

Figure 36. Bcl6 inhibition reduces IL-21 production

Il21 transcript level **(A)** and concentration of IL-21 from the accumulated supernatant **(B)** of naïve CD4 T cells cultured with different concentrations of 79-6 for 4 days. **(A and B)** The data represent at least two experiments. **(A)** Results are shown as the mean ± SEM. * P<0.05, ** P<0.01, and *** P< 0.005 as determined using two way ANOVA. **(B)** Results are shown as the mean ± SEM. * P<0.05, ** P<0.01, and *** P< 0.005 as determined using Student's t-test.

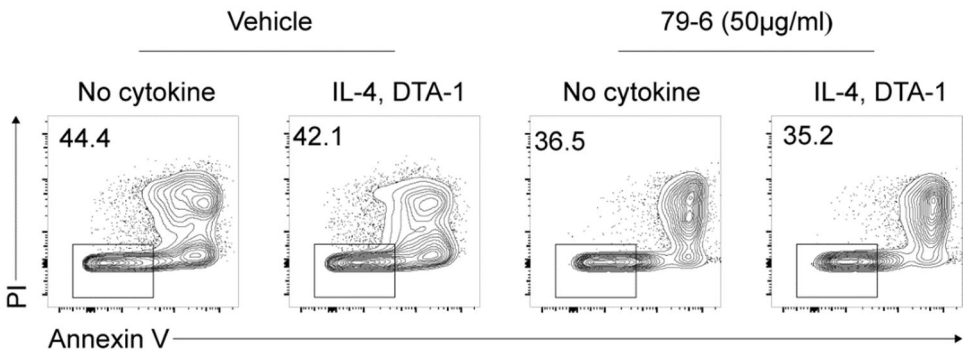

Figure 37. Bcl6 inhibition does not induce significant cellular toxicity

IL-4 and DTA-1 stimulated naïve CD4 T cells cultured with 79-6 and followed by staining of Annexin V and PI. The data represent two experiments.

I next determined the role of Tfh cells in tumor growth inhibition by DTA-1. In the CT26 tumor model, administration of 79-6 significantly reduced the frequencies of Tfh cells in DTA-1-treated mice and IL-21–producing CD4 T cells were also diminished in these mice **(Figure 38)**.

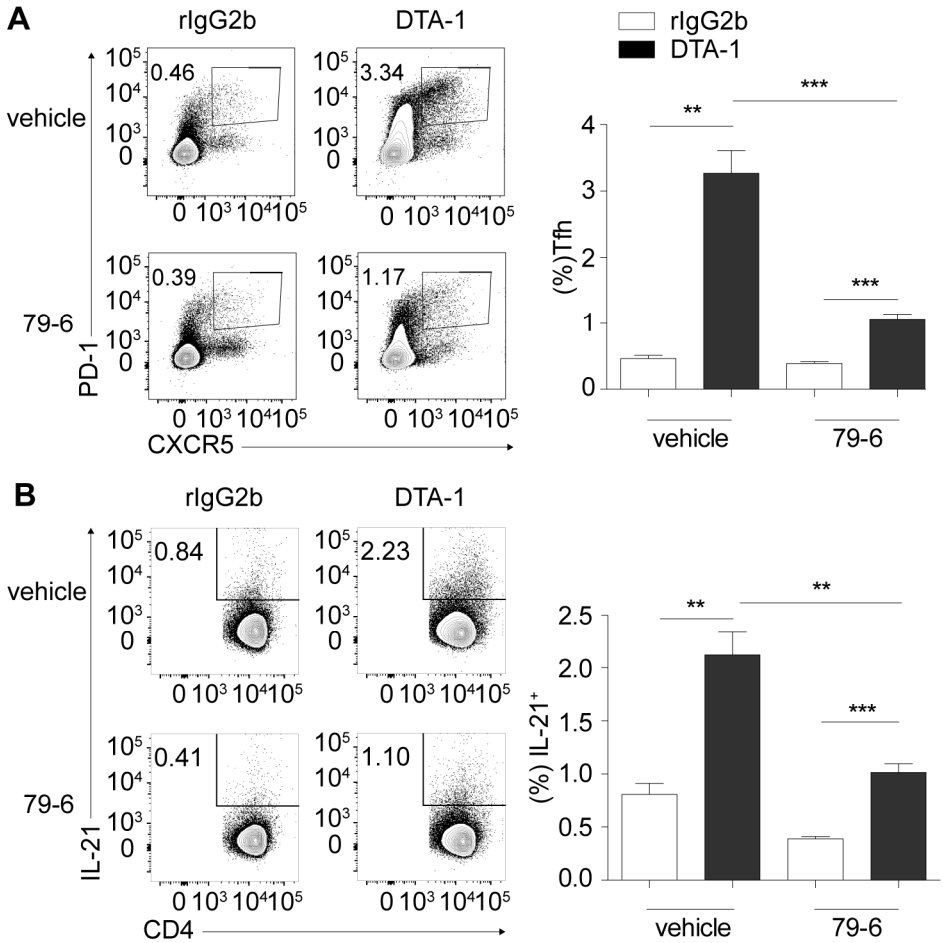

Figure 38. Bcl6 inhibition reduces the frequency of DTA-1 induced IL-21$^+$ Tfh cells *in vivo*.

BALB/c mice were s.c. injected with CT26 cells, and 5 days later, they were i.p. injected with DTA-1 or rIgG2b control antibodies. After 3 days of antibody administration, the Bcl6 inhibitor 79-6 or the vehicle control were i.p. treated daily for 5 days. **(A)** CXCR5 and PD-1 expression from the CD4

T cells in TdLNs of DTA-1 and 79-6 treated mice is shown in contour plots (left) and graph (right). **(B)** IL-21–secreting CD4 T cells from TdLNs of DTA-1 and 79-6 treated mice are shown in contour plots (left) and graph (right). **(A and B)** The data represent at least two experiments. Results are shown as the mean ± SEM. * P<0.05, ** P<0.01, and *** P< 0.005 as determined using Student's t-test.

Consequently, tumor growth inhibition by DTA-1 was reversed by 79-6 treatment **(Figure 39)**.

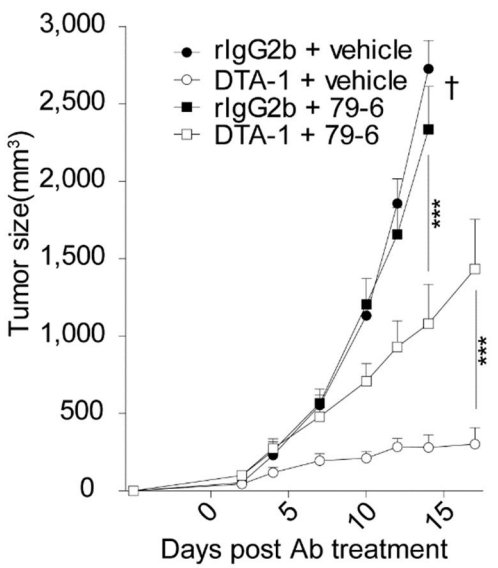

Figure 39. Bcl6 inhibition compromises antitumor effect of DTA-1.

Tumor size from CT26 tumor bearing mice treated as in **figure 29** was monitored 2 days after DTA-1 injection. The data represent at least two experiments. Results are shown as the mean ± SEM. * P<0.05, ** P<0.01, and *** P< 0.005 as determined using two way ANOVA.

To confirm the specific requirement of Tfh cells in DTA-1-induced antitumor immunity, I used *Bcl6*$^{fl/fl}$*Cd4*^{Cre} mice in a MC38 and TC-1 tumor model. As expected, *Bcl6*$^{fl/fl}$*Cd4*^{Cre} mice showed reduced frequencies of IL-21^{+} Tfh cells in the TdLN **(Figure 40)**.

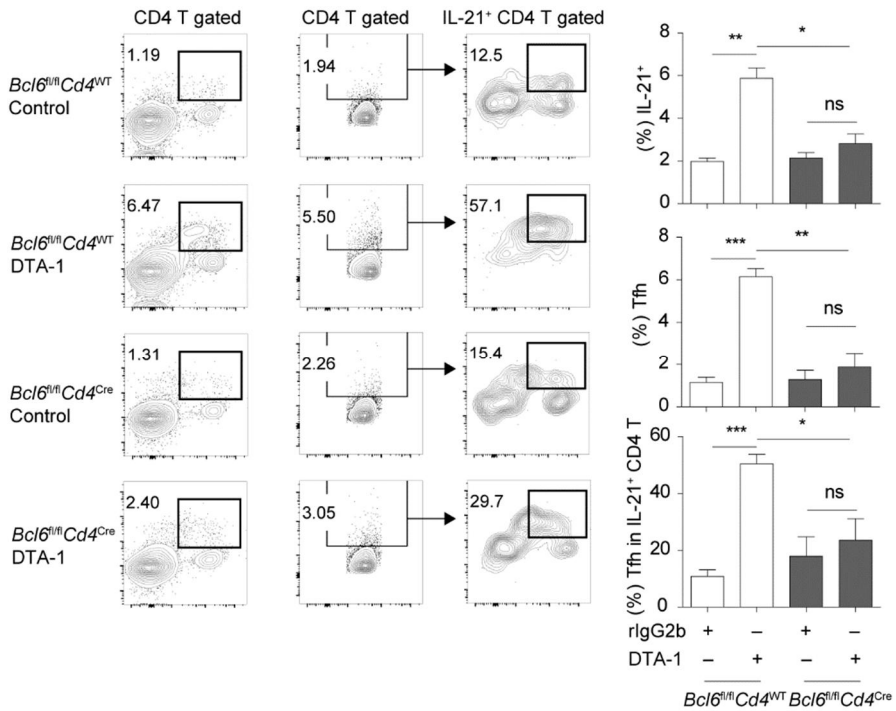

Figure 40. Deletion of Bcl6 in CD4 T cells results in reduced induction of IL-21$^+$ Tfh cells by DTA-1.

Bcl6$^{fl/fl}$*Cd4*^{cre} mice were s.c. injected with TC-1, and DTA-1 was treated after 7 days. Another 7 days later, TdLNs of tumor bearing mice were harvested and analyzed for the expression of IL-21, CXCR5 and PD-1 on CD4 T cells. The data represent two independent experiments and results are shown as the mean ± SEM. * P<0.05, ** P<0.01, and *** P< 0.005 as determined using Student's t-test.

Bcl6 deficiency in T cells resulted in impaired DTA-1-induced inhibition of tumor growth compared to the control mice in TC-1 and MC38 models **(Figure 41)**.

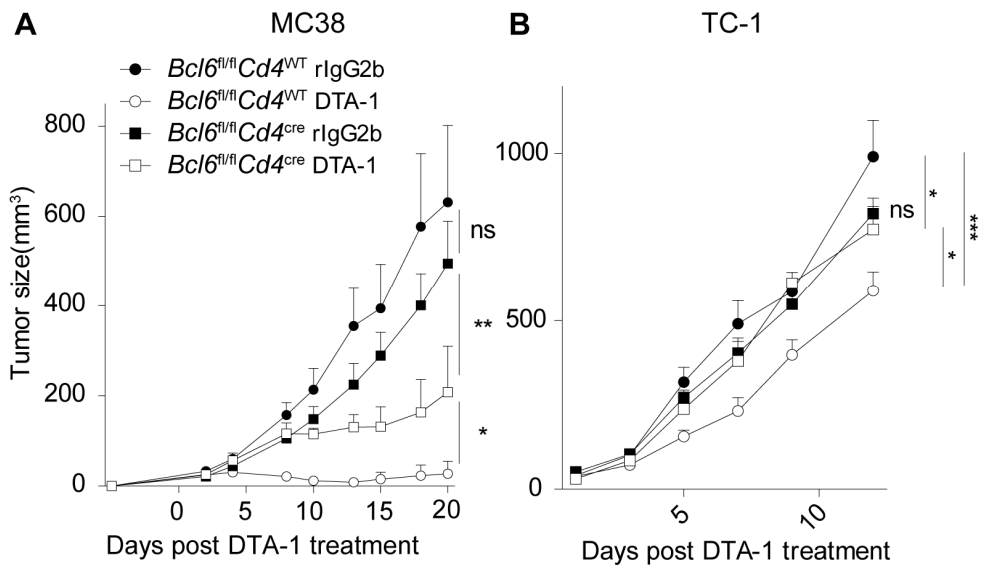

Figure 41. Antitumor effect of DTA-1 in *Bcl6*$^{fl/fl}$*Cd4*^{Cre} mice

Tumor size of *Bcl6*$^{fl/fl}$*Cd4*^{cre} mice which were s.c. injected with MC38 **(A)** and TC-1 **(B)** and DTA-1 or rIgG2b control antibodies were i.p. treated 5 days after tumor cell inoculation. **(A and B)** The data represent two independent experiments. Results are shown as the mean ± SEM. * $P<0.05$, ** $P<0.01$, and *** $P< 0.005$ as determined using two way ANOVA.

Although *Bcl6*$^{fl/fl}$*Cd4*^{Cre} mice also have a Bcl6 defect in CD8 T cells, the CD8 T cells in these mice produced sufficient IFN-ɣ in response to IL-21 **(Figure 42)**. Collectively, these results suggest that Tfh cell are required for the antitumor activity induced by DTA-1.

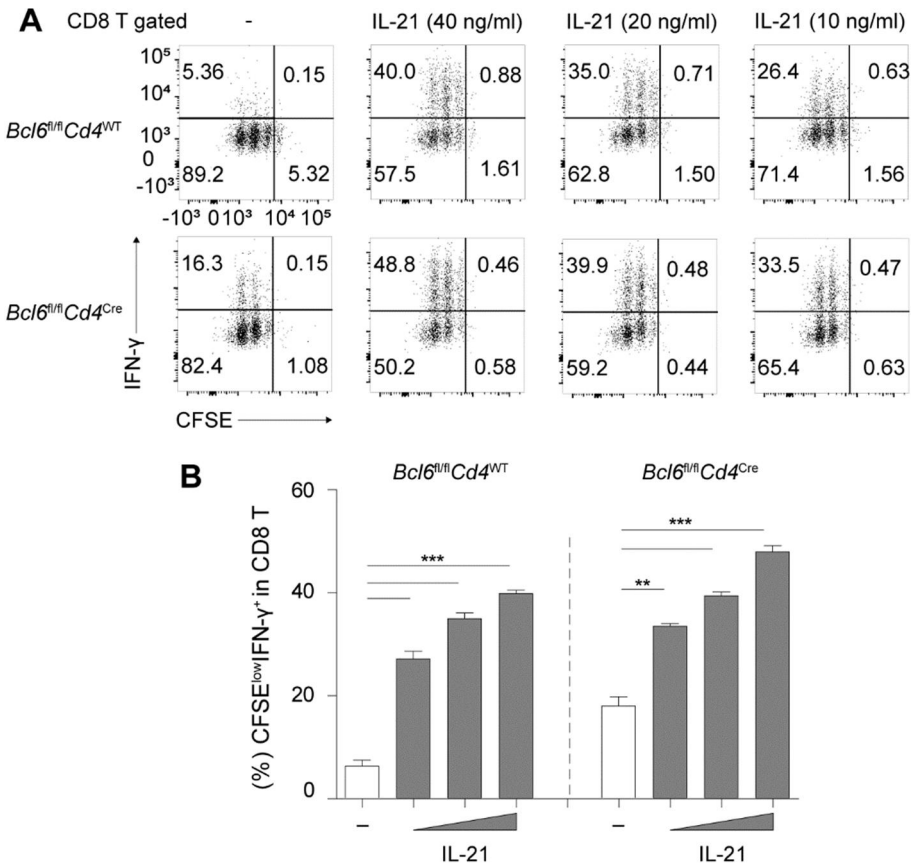

Figure 42. *Bcl6*$^{fl/fl}$*Cd4*^{Cre} CD8 T cells respond to IL-21 *in vitro*.

CFSE-labeled CD8 T cells from *Bcl6*$^{fl/fl}$*Cd4*^{Cre} mice were cultured with soluble αCD3 antibody and T cell-depleted antigen presenting cells (APCs) at the indicated ratio of IL-21 cytokine are shown. CFSE dilution and IFN-ɣ production from CD8 T cells are shown in dot plots **(A)** and graph data **(B). (A and B)** The data represent two independent experiments and results are shown as the mean ± SEM. * $P<0.05$, ** $P<0.01$, and *** $P< 0.005$ as determined using Student's t-test.

Chapter 4. Conclusion

GITR agonism has been a promising strategy for cancer immunotherapy and extensive works have demonstrated its cellular mechanism in the context of Treg and CD8 T cells (14,16,18,19,55). Distinguished from other studies, my research focused on cytokines produced by CD4 T cells, and I had previously shown that the GITR agonistic antibody exerts antitumor activity in an IL-9–dependent fashion (20). However, the neutralization of IL-9 did not completely reverse the increased CTL activity induced by DTA-1, suggesting that DTA-1 has multiple arms of antitumor activity. In this study, I found that IL-21 was essential for antitumor CTL responses and subsequent tumor growth inhibition by DTA-1 treatment, and production of IL-21 was mainly achieved by Tfh cells in an IL-9–independent and antigen specific manner. Moreover, I demonstrated that GITR costimulation promoted IL-21 expression in CD4 T cells with IL-4, via c-Maf. Thus, I have unveiled an underlying mechanism of DTA-1–induced antitumor immunity and its molecular mechanism for the induction of IL-21–producing CD4 T cells **(Figure 43)**.

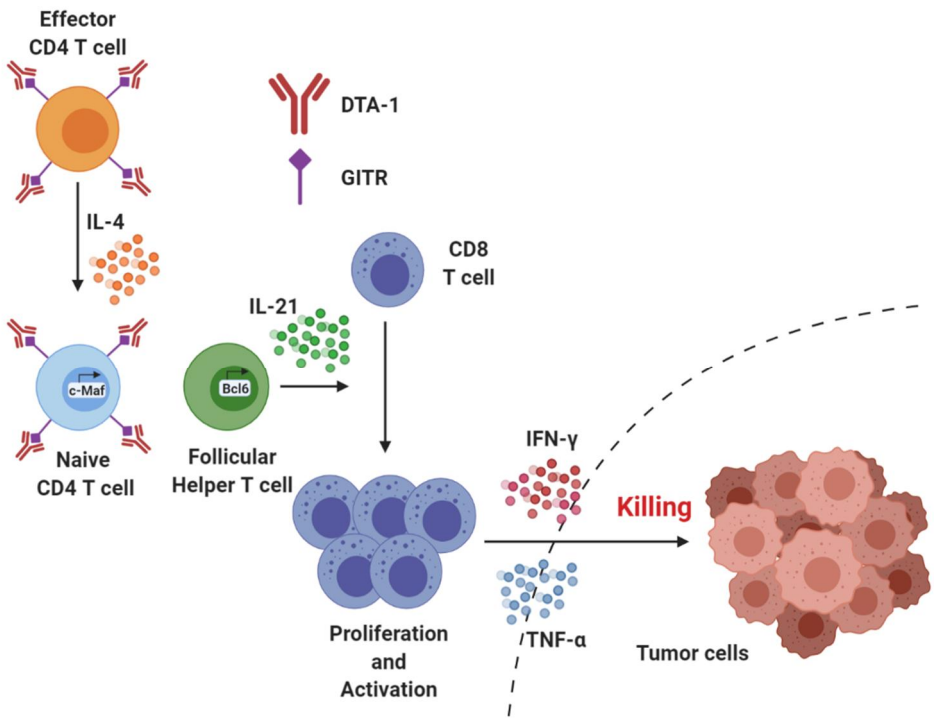

Figure 43. A schematic illustration of IL-21–producing follicular helper T cell-mediated antitumor immunity induced by GITR co-stimulation.

GITR costimulation induced IL-21–producing Tfh cells through IL-4, c-Maf signaling pathway. Increased IL-21 signal directly enhanced effector molecule production in CD8 T cells and resulted in tumor regression.

Previous works have demonstrated the mechanism of GITR agonism and found that CD4 T cells and mature B cells are responsible for antitumor immunity induced by DTA-1 (12,44). Depletion of CD4 T cells inhibited the activation of CD8 T, NK and B cells after DTA-1 treatment and resulted in uncontrolled tumor growth *(12)*. Another study demonstrated that DTA-1 treatment significantly increased B cell responses in tumor bearing mice and failed to induce antitumor immunity in mature B cell deficient JHD mice with low CTL activity (44). On the basis of the current study, I suggest that Tfh cells act as a means to account for the role of CD4 T cells and B cells in DTA-1 induced antitumor immunity. Although I focused on the effects of DTA-1 induced IL-21–producing Tfh cells on CD8 T cells in this study, their effects on mature B cells remain for further studies.

Several studies have previously reported the positive role of GITR on Tfh cell generation. GITR signaling plays a crucial role in the pathogenesis of chronic LCMV infection and collagen-induced arthritis by upregulating Tfh cell responses (56,57). However, the role of GITR on Tfh cells in tumor microenvironments has not been defined yet. Consistent with other models, GITR costimulation increased antigen-specific Tfh cells in

tumor–draining lymph nodes and tumor tissues. Although recent clinical studies have shown that the frequency of circulating Tfh cells was increased in cancer patients, the role of Tfh cells in tumor immunity has been controversial (42,58,59). I assume that the role of Tfh cells in tumor immunity is determined by the types of tumors. In case of solid tumors, tumor infiltrating Tfh cells contribute to the formation of secondary lymphoid structure adjacent to tumor site and activate antitumor immune responses, including humoral immunity and CTL responses (40). However, Tfh cells help tumor growth in diffuse large B cell lymphoma (DLBCL). In case of DLBCL, tumor cells are B cells that grow and activate from the help of Tfh cells (42). In this context, I suggest that Tfh cells as antitumorigenic participants since they potently activated antigen-specific CD8 T cells in colon carcinoma models. Furthermore, Bcl6-deficient model confirmed the requirement of Tfh cells in DTA-1 induced tumor rejection.

I observed that $CD62L^{-}CD44^{+}$ effector CD4 T cells and $CD62L^{+}CD44^{-}$ naïve CD4 T cells each produced less IL-21 than the coexisting naïve and effector CD4 T cells did upon GITR costimulation which was mediated by IL-4 signal transduction in naïve CD4 T cells. I assume from this result that, since most of effector CD4 T cells are

differentiated cells, effector CD4 T cells are refractory to re-differentiate into IL-21–producing T cells. Also, IL-4 produced by GITR costimulated naïve CD4 T cells is not sufficient for IL-21 induction. Both effector and naïve CD4 T cells are responsible for optimal IL-21 production by GITR costimulation: effector CD4 T cells as IL-4 providers and naïve CD4 T cells as IL-21 producers. Recent clinical trial reported that human GITR agonistic antibody was not sufficient to mediate substantial clinical responses (19). On the basis of my results, the limited efficacy of GITR–targeting monotherapy might be due to an insufficient portion of naïve CD4 T cells to produce IL-21 in the patients with the late stage cancer.

IL-4 is a typical type 2 cytokine and plays a crucial role in humoral immune responses. Previous studies have shown that Tfh cells are also IL-4 producers, although Th2 cells are considered the main producer of IL-4 (60-63). Indeed, in a helminth infection model, IL-4 secretion was restricted to Tfh cells in an ICOS–dependent manner (64). On the other hand, a recent study has also proposed IL-4-committed Tfh cells as precursors of short-term effector Th2 cells upon house dust mite challenge (60). My findings demonstrated that IL-4 was required for the optimal induction of IL-21–producing Tfh cells upon DTA-1 treatment and human naïve CD4 T cells

also produced IL-21 in the presence of IL-4 upon hAITR costimulation. In addition, I observed that the frequency of $CXCR5^{+}PD\text{-}1^{+}$ Tfh populations was not increased in CT26 tumor bearing IL-4 receptor knockout mice treated with DTA-1. To my knowledge, this is the first report to reveal the role of IL-4 as an inducer of IL-21–producing Tfh cells. Further studies are needed to define a specific requirement of IL-4 and GITR costimulation for Tfh cell development and whether agonistic anti-hAITR treatment also induces $IL\text{-}21^{+}$ Tfh cells and has antitumor activity through this population in human cancer patients.

The roles of c-Maf in Tfh cell development and IL-21 expression have been previously described. C-Maf directly binds to the promoter and CNS-2 regions of IL-21 gene loci and activates IL-21 expression in CD4 T cells (65). Upon IL-21 induction *in vitro*, c-Maf was transiently upregulated among IL-21–regulating transcription factors. ShRNA targeting *Maf* gene indicated that c-Maf mediated IL-21 production in naïve derived CD4 T cells with IL-4 and DTA-1. Whether GITR costimulation directly regulates c-Maf expression remains to be elucidated.

My study revealed a novel mechanism by which GITR costimulation inhibits tumor growth through the induction of IL-21–

producing Tfh cells. IL-4 plays an important role in IL-21 production in Tfh cells upon GITR costimulation via c-Maf. Therefore, my findings provide a fundamental basis for the use of GITR agonists to treat cancer and suggest IL-21 as a surrogate marker for its effectiveness.

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국문초록

암의 치료에 대한 면역학적 접근은 세계적으로 많은 연구가 진행되고 있으며, 항 CTLA-4 항체, 항 PD-1 항체와 같은 면역관문 억제제는 악성흑색종 치료에 뛰어난 효과를 나타낸다는 사실이 밝혀졌다. 면역관문 억제제의 항암치료는 기존의 항암치료 방법을 상회 하는 효과를 보였으나, 모든 암 환자에게 효과가 있는 것은 아니었다. 이에 면역관문 억제제가 치료하지 못하는 환자들에게 효과가 있는 새로운 면역항암 치료에 관한 연구가 계속되었고, 대안으로 공자극 분자 자극 항체에 관한 연구가 진행되었다. 공자극 분자는 면역 세포를 활성화하는 신호를 전달하게 되는데, 공자극 분자 자극을 통해 암을 사멸시킬 수 있는 면역 세포가 활성화될 경우 체내 면역 세포를 이용한 항암효과를 기대할 수 있다. 현재 공자극 분자 중 가장 활발히 연구가 진행되고 있는 GITR (glucocorticoid-induced tumor necrosis factor receptor-related protein)은 마우스 모델에서 매우 효과적인 항암효과를 보였으나, 어떠한 기전으로 항암효과를 나타내는지에 대한 연구는 아직 미흡한 수준이

었다. 이에 항 GITR 자극 항체의 항암기전을 확인하고자 실험을 계획하였으며 이번 연구를 통하여 항 GITR 자극 항체의 항암기전으로 Follicular Helper T 세포의 IL-21 생산 유도를 통해 항암면역작용이 일어난다는 사실을 확인하였다. 먼저 암을 주입한 마우스에 항 GITR 자극 항체를 복강투여할 경우 암 주변 림프절에서 IL-21을 발현하는 CD4 T 세포의 증가가 일어나는 것을 확인하였고, 항 IL-21 수용체 항체를 이용하여 IL-21 신호를 차단하였을 때, GITR 자극에 의한 항암효과가 줄어드는 것을 확인하였다. 여러 CD4 T 세포의 아형 중 GITR 자극에 의해 IL-21을 발현하는 아형은 어떤 것일지 살펴보았고, CXCR5와 PD-1을 발현하는 follicular helper T 세포라는 것을 확인하였다. 또한, GITR 자극에 의한 IL-21 발현에 IL-4가 반드시 필요하며 이 과정에서 c-Maf의 신호전달경로를 거치게 된다.

다음으로 GITR 자극에 의해 발현된 IL-21이 어떠한 세포에 영향을 미쳐 항암효과를 나타내는지 확인하였다. GITR 자극 후 IL-21이 생산된 뒷 시점을 조사하였을 때, 자연살해세포는 활성화되어 있지 않았지만, CD8 T 세포가 활성화되어 있음을 확인하였다. 이에 IL-

21 신호전달을 차단한 경우 CD8 T 세포가 사이토카인 분비 및 암 세포에 대한 세포독성 효과가 감소하는 현상을 확인하였다. 본 연구를 통해 GITR 자극이 암 환경에서 Tfh 세포에 의한 IL-21 발현을 유도하여 항암면역 효과를 나타낸다는 작용기전을 최초로 밝혔으며, 이에 IL-4의 역할을 규명함으로써 IL-4가 IL-21 유도에 기여하는 바를 밝혀 GITR 자극 항체의 임상적 적용 시 확인할 수 있는 새로운 마커를 제시하였다는 것에 의의가 있다.

주요어

GITR 자극 단클론 항체, IL-21, Follicular helper T 세포, IL-4, c-Maf, 세포 독성 T 세포

학번

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감사의 말

7년 간의 대학원 생활을 마무리 하는 졸업논문을 작성하게 되어 기쁜 마음입니다. 제 졸업논문을 성공적으로 마무리하게 될 수 있었던 것은 저 혼자만의 힘으로는 부족한 일이었을 것입니다. 가장 먼저 좋은 주제로 연구를 진행할 수 있는 기회를 주신 교수님께 감사드립니다. 또한, 박사학위 졸업논문을 쓰게 되며 논문심사 과정에서 논문 작성에 많은 도움을 주신 여러 교수님들께 감사의 말씀 전합니다. 특히나 논문 작성에 많은 도움을 주시고, 논문심사 위원장까지 맡아주신 정연석 교수님께 감사의 말 전합니다. 논문 투고 및 수정 과정에서 많은 아이디어를 주시고, 꼼꼼하게 챙겨주신 김병석 선배님께 감사의 말 전합니다.

다음으로, 많은 시간을 함께한 우리 실험실 가족들에게 감사의 말 전합니다. 신입생으로 들어와 아무것도 모르는 상태부터 실험하는 방법을 기초부터 알려주고, 후에 주제를 잡고 나서도 어려움이 있을 때마다 같이 논의를 해주신 김일규선배님께 감사의 말 전합니다. 실험실에 처음 들어왔을 때, 실험실에서의 생활에 대한 방향성을 보고 배울 수 있게 해준 박영준 선배님께 감사의 말 전합니다.

실험실 선배로 많은 본보기를 보여주었던 형석이형에게도 이 자리를 빌어 감사의 말 전합니다. 실험적으로 많은 토론도 했고, 특히나 후반부엔 함께 과제를 쓰면서 호흡을 맞춘 보영누나에게 정말 많은 것들 배울 수 있었던 점 감사드립니다. 학부 동기이자 실험실 선배인 은아에게도 감사의 말 전합니다. 항상 제일 일찍 출근해서 늦게까지 연구에 매진하는 모습을 옆에서 보며 많은 것을 배웠습니다. 학부 때부터 동기로 시작해서 실험실에 와서도 함께 생활하고, 특히 한학기 먼저 졸업한 사람으로 많은 도움을 준 광수에게 정말 고맙고 미래에 대한 큰 결심한 것 축하하고 높은 자리까지 올라가길 진심으로 응원합니다. 실험실 동기로 들어온 인수형에게 항상 고마웠던 마음 여기에서 전합니다. 형이지만 늘 편하게 대해줘서 함께 하는 실험실 생활이 즐거우면서도 많이 의지가 되었습니다. 최근에 물어볼 일들이 많이 생기곤 하는데, 본인이 바쁜 와중에도 늘 친절히 알려주는 모습을 보며 많이 배웠습니다. 하나뿐인 박사 후배 정미에게 감사의 말 전합니다. 긴 시간은 아니지만 함께 실험 했을 때, 대학원에 들어온 지 얼마 안 된 시점이었는데도 금방 배우고 너무나 잘 해 주어서 정말 많은 도움이

되었습니다. 가끔씩 약과 관련해서 물어보거나 도움을 청할 때도 많은 도움을 줬던 것도 항상 감사합니다. 실험실 공식 막내 태승이에게도 이 자리를 빌어 감사의 말 전합니다. 실험실에 와서 실험만 배우기도 힘들었을 텐데, 야무지게 행정관련 일들 처리 하느라 정말 고생 많았고, 덕분에 우리 실험실이 잘 유지될 수 있었습니다. 마지막에 같이 졸업 준비하면서 고생이 많았는데, 이제 잘 마무리 되었으니 취업한 회사에서 새로운 사람들 만나서 즐겁게 잘 지내길 바랍니다.

다음으로 힘든순간에도 버팀목이 되어준 우리 가족들에게 감사의 말을 전합니다. 먼저, 할아버지 할머니께 감사의 말씀 전합니다. 바쁘다는 이유로 자주 연락드리지 못하는 못난 손자도 항상 아껴주시고 걱정해주셔서 감사드립니다. 할아버지 할머니 두 분 모두 건강히 오래오래 사시면서 손자 손녀들을 비롯한 우리 가족 모두 행복하게 잘 살아가는 모습 지켜봐주시길 부탁드립니다. 다음으로 우리 집안의 가장으로 중심을 잡아준 아버지에게 감사의 말씀 전합니다. 아버지께서 내려주신 튼튼한 뿌리 덕분에 무사히 박사학위를 받을 수 있었습니다. 그리고 늘 세심하게 보살펴 주신 어머

니에게 감사의 말씀 전합니다. 어머니로, 또 먼저 박사학위 받아본 선배로 해주신 많은 말씀들이 힘든 순간에 큰 도움이 되었습니다. 새로운 결정을 앞두고 고민이 많을 형에게도 고마움의 말을 전합니다. 대학원을 다니게 되고, 나와서 살게 되면서부터 자주 보진 못했지만, 제게 필요한 건 없을까 챙겨주던 형의 마음 씀에 항상 고마웠습니다. 이제 큰 결정을 앞두고 있는데, 신중하게 고려한 선택이니 분명 좋은 결과가 있을거라 생각합니다. 그리고 장인어른과 장모님께도 늘 가지고 있던 감사한 마음 이 자리를 빌어 전합니다. 아직 아무것도 결정된 것이 없는 대학원생이었던 저에게 소중한 따님과의 결혼을 허락해주셔서 정말 감사드립니다. 이제 졸업하고 학생의 신분을 벗어나는 만큼, 기대해주신 것에 부응할 수 있도록 성혜와 행복한 삶 꾸며가는 모습 보여드리도록 하겠습니다. 마지막으로, 사랑하는 부인, 성혜에게 가장 큰 고마움 전합니다. 대학원신입생과 회사 신입사원으로 처음 만나 결혼하고, 졸업하는 지금까지 항상 곁에서 힘이 되어 주어 정말 고맙습니다. 처음 만날 때부터 하던 졸업하겠다는 이야기를 6년이 지난 지금에서야 지키게 되었습니다. 함께 해준 덕분에 힘든 순간도 긍정적으

로 생각하며 지나갈 수 있는 힘을 키울 수 있었고, 앞으로 고난이 온다 해도 함께라면 잘 헤쳐나갈 수 있을 것 같다는 자신감을 갖게 되었습니다. 적극적으로 믿어주고 지지해 주었기에 미국에 함께 가겠다는 큰 결심도 내릴 수 있었습니다. 그동안 우리 집의 가장으로서 책임 때문에 어깨가 많이 무거웠을 텐데 이제는 함께 나눠지도록 해요. 정말 고생 많았고, 고맙고 사랑합니다. 제일 마지막으로 우리 고양이 모모에게도 감사함과 미안함의 말 전합니다. 그동안 바쁘다고 많이 못놀아줘서 미안해. 이제 새로운 낚시대로 더 신나게 놀아줄게. 쫓아다니면서 우다다도 많이해줄게. 아프지 말고 오래오래 함께 살자 모모야. 사랑해.

많은 분들의 도움 덕분에 지금 이 자리에 올 수 있었단 사실을 너무나 잘 알고 있습니다. 미처 감사의 말에 올리지 못한 많은 분들께 감사의 말씀을 전합니다. 학부를 졸업하고 실험실 생활을 하며 앞으로 살아가는 자세에 대하여 많은 고민을 하며 혼자만의 힘으로 이룰 수 있는 것은 없으며, 끊임없이 노력하고 겸손한 자세로 정진해야 한다는 것을 배우게 되었습니다. 제가 배운 바를 항상 잊지 않고 앞으로 나아가는 모습 보여드리도록 하겠습니다.

감사합니다.

2020년 1월 14일 고충현 올림.